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Genetic Diversity of Genotype VII, and Virulent Newcastle Disease Virus (NDV), Determined by Cleavage Site Fusion Protein, and Heterogeneity with Commercial Vaccine Strains

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THE objective of this study was to Fig. out the most common velogenic NDV strain responsible for virus outbreaks in Sulaimani, Iraq's chicken farms. Even though he implementation of a thorough vaccination program for poultry farm, the infection has spread to commercial broiler. The genetic discrimination between the NDV strains and pathological samples was made using the fusion (F) protein cleavage site. The velogenic NDV strain-specific motif (^{112}R-R-Q-K-R-.F^{117}) for the F protein cleavage site was identified in two NDV isolates (NDV/M/20 and NDV/M2/20). Furthermore, these isolates were identified as belonging to class II, genotype VII, by phylogenetic analysis based on a partial sequence of the F protein gene, in contrast to the commercial vaccine strains that were often employed, which were genotype II of class II. This is the first research to find such NDV strains in commercial chicken farms in Sulaimani province. To ensure proper administration and reaction to emerging strains, field vaccination protocols should be evaluated regularly, also to decrease the potential of epidemics of poultry Newcastle disease, stringent biosecurity measures must be implemented.

Keyword: Fusion gene, Very virulent, Genotyping, Phylogeny tree, Prevalent.

Introduction

Newcastle disease is a highly contagious and transboundary disease that affects a wide range of avian species. The outbreaks of deadly diseases that affect commercial chicken flocks are mostly caused by it, They cause large annual economic losses for the industry and still become endemic [1, 2]. viral NDV infection can cause fatal clinical manifestation include diarrhoea, respiratory ,and neurological system disorder, decrease egg production, misshape ,and egg quality [3, 4]. Additionally, NDV lead to in haemorrhagic lesion in the proventriculus, trachea, and gut [5-7]. NDV is a member of the paramyxoviridae family, and the avulavirus genus [8-10] A non-segmented RNA with a negative sense length approximately 15 kb, compose a majority of the NDV genome [11, 12]. The six gene segments that make up the genome are inserted at the 3' and 5' termini, between the Leader and Trailer viral polymerase promoter.

(3'NP,PM,F,HN,L5') which stimulate

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transcription, and replication of RNA [13]. this genome structure consist of two non-structural ,and six structural viral proteins, non-structural include: V, and, protein and structural protein include : nucleocapsid protein-NP, phosphoprotein-P, matrix protein-M, fusion protein-F, hemagglutinin neuraminidase-HN, large protein-L [11]. NDV is susceptible to chemical and disinfectants such ether, phenolic acid, and oxidizing agent like chlorhexidine, 6% sodium hypochlorite, and the virus inactivated at 56 ˚C for three hours and/or PH2<2. (OIE 2011). There are three main pathotype of Newcastle disease, depending on clinical sings on chicken, and mortality of embryo: lentogenic (apathogen) type, and velogenic, mesogenic (pathogen) type. [14]. Based on the analysis of the target area F gene sequence, which is found in DNA sequences with positions 47–421, class I and class II NDV strains are divided [15]. class I include the majority of lentogenic type isolated from water birds, while Class II it has higher genetic diversity and, affecting different avian hosts and identified many genotypes, which comprise virulent and avirulent strains [16, 17].

The NDV pathogenicity is strongly affected by the cleavage location of the F protein. Cellular proteases cleave an inactive F0 precursor to generate an active F protein, resulting in the formation of the disulfide-linked F1 and F2 subunits [18]. For viral infection, F1 and F2 are essential. The dual-basic amino acids in the cleavage motif of the lentogenic strains can only be broken down by the trypsin-like proteases found in the respiratory and digestive systems[19, 20], whereas velogenic strains have multiple types of basic amino acids at the cleavage site that are recognized by numerous furin-like proteases. As a consequence of systemic infection, virulent strains are lethal for chickens [21, 22].

In this review, we look at the present diversity of NDV genotypes in Iraq and evaluate the implications of the genotype mismatch between circulating field strains and vaccine strains for NDV management in the country, furthuremore The viruses were genotyped by phylogenetically analysis of the F-gene sequence and pathotyped according to their F-gene cleavage site sequence.

Materials and Methods

Sample preparing

Samples were obtained from poultry farms in two geographical region (Dokan, and Sharbazher) as shown Green zone in (Fig. 1) in Sulaimani province between June and December 2020. From 10 vaccinated broiler flocks, field tissue samples of several internal organs (Cecal tonsil, proventriculus, gizzard, intestine, and trachea) were taken. The birds are between the ages 23-30 days, and mortality rate between 35-80%. with post mortum lesion in liver, gizzard, proventiculus and ceacal tonisl (Fig. 2).

Viral RNA Extraction.

NDV RNA was extracted from tissue samples from each flock and in PH 7.0-7.4 sterile phosphate buffered saline, pooled, and homogenized. According to the manufacturers' instructions total RNA mini kit (Genaid, Rep. Korea). Pellet RNA was immediately used in RT-PCR after being eluted in 100 µl of eluting buffer.

RT and polymerase chain reaction (RT-PCR)

For the current study was used two sets of primers were, the first one NDV-F (5'-GGTGAGTCTATCCGGARGATACAAG-3') and NDV-R (5'-TCATTGGTTGCRGCA ATGCTCT-3') used for screening all suspected NDV farms, this primer generated 202 bp [23]. and second set the primer M1055 (F-5-GCTGATCATGAGGTTACCTC-3),andtheprimer F508 (R-5-AGTCGGAGGATGTTGGCAGC-3), They were employed to amplify the fusion protein cleavage site and the target region of the fusion gene, This set of primers generated a 697 bp fragment between the nucleotides 508 of the fusion (F) gene and 1055 of the matrix (M) gene [24]. SuprimeScript RT-PCR Premix has been used to amplify target of the F gene's sequence. This kit provides a complete solution for quick, efficient, and dependable single tube one-step RT-PCR. (Genet Bio. Republic Korea). For the polymerase chain reaction, PCR was mixed to a final volume of 20 µl. The thermal cycler was used for amplification (Hercuvan Lab Systems, Cambridge, UK). The following processes were used in the PCR: 30 min of cDNA synthesis at 50 °C, followed by 10 min of initial denaturation at 95 °C, 40 cycles of 95 °C for 30 sec, 30 sec of annealing at 56 °C, 30 sec of extension at 72 °C, and 10 min of final extension at 72 °C. The ND primer and F508 primers each had their primer extension temperatures set at 30 and 40 seconds, respectively.

Agarose Gel Electrophoresis

Ten µL of the PCR products that had been amplified were run on a 1% agarose gel and stained with 3 µl safe dyes (EURx, Banino, Poland), in a mixture of trisacetate-EDTA (TAE) 1x buffer. The PCR result passed gel electrophoresis and was observed with a gel documentation system (Uvitec, Cambridge, UK)

Sequences analysis

To determine the virus's ancestry, the PCR results were sequenced using the Sanger sequencing method (Macrogen. Co. Republic Korea). Following that, using the Clustal W technique, the results were aligned with the NDV reference sequences available in NCBI GenBank. Subsequently, the neighbor-joining method MEGA X [25, 26] was used to determine phylogenetic relationships using the tamura 3 parameter model with 1000 bootstrap replicates [27].

Results

Identification

To identify viruses, RT-PCR is utilized. Six of the ten samples tested were found to be positive by RT-PCR analysis, with a band of 202 bp for primer (ND-F & ND-R), and 697 bp for primer (M1055 & F508) (Fig. 3). Both strains (NDV/M/20 and NDV/M2/20) have had their partial F gene sequences (462 bp) submitted to the International Nucleotide Sequence Database (INSD), which is part of the National Center for Biotechnology Information (NCBI/Genebank) under the accession number (MW491883.1 and MW491884.1).

NDV Genotyping and sequence analysis

Tow field isolated viruses (NDV/M/20 and NDV/M2/20) are related to genotype VII, according to the results of the phylogenetic analysis of the partial sequences of the selected strains(Fig. 4). Also, the results indicated that the F gene sequence of strains (NDV/M/20 and NDV/M2/20) was closely related to that of the Indonesian (MN557407), Pakistan (MT920211), Turkey (KT585633.1), and Iran (MH588684.1) NDV strains, with amino acid identities (98.70 -98.05, 98.05 - 97.40, 96.21- 95.45, and 96.75-96.10) respectively(Table 1). In addition in phylogenic tree demonstrated that all commercial vaccine strains were heterologous with field strains and belonged to genotypes I and Genotype II (Fig. 4). furthermore, the analysis found that 96.35% of the field isolates were identities with each other. Depending on the identity of the nucleotide and amino acid, the similarity ranged from 80.50 to 97.63% and 80.85 to 99.11%, respectively, when compared to other prior Iraqi strains identified between 2012 and 2019 (Table 2 & Fig. 5). based on the R-R-Q-KR-F motif that inludes multiple basic amino acids observed in the amino acid sequence of the F protein proteolytic cleavage site, field virus strains were classified as virulent NDV strains , Furthermore, 16 Iraqi strains with virulence and 6 Iraqi strains with lentogenic were found in the cleavage site region (Fig. 5). Multiple sequence alignments of the amino acid circulatory vvNDV genotype and commercial vaccines strain revealed that 13 amino acid substitutions in fusion proteins were detected (Fig. 6).

Discussion

Newcastle disease virus (NDV), a highly lethal avian disease, poses a serious danger to the poultry industry around the world. Despite Iraq is implementing a comprehensive NDV management program that includes vaccination bird with a commercial vaccine strain like (clone 30, B1, Lasota) NDV continues to be an endemic disease in the country. This could act as a global distributor of velogenic pathotypes across regional and international borders as wild birds may act as a reservoir for avirulent NDV strains that cause virulent diseases in chicken [28]. Phylogenetic and genetic studies of the F gene sequences, the current study demonstrated that the continuing circulation of velogenic NDV of subgenotype VII, among broiler chickens in Iraq, as shown in (Fgure4) velogenic NDV more prevalent than lentogenic NDV. Alignment of the two field isolates' F gene partial amino acid sequences with other published NDVs from different genotypes. The isolates' cleavage site was found to be at (^{112}R-R-Q-K-R-F^{117}), This is typical of virulent velogenic NDV strains because it contains several basic amino acids that are identified by ubiquitin-like proteolytic enzymes. Virulent strains cause systemic infection in poultry, which is deadly [21, 22]. The presence of a phenylalanine (F) residue at position 117 is also significant, this result are compatible with high mortality of of the field study are used for investigation. Furthermore, all commercial NDV vaccines, such as (ulster/I, (Fl)44083, strain v4, avinew) strain genotype I, however, (Clone-30, strain-B1, Lasota, and Komarov) strain genotype II, a considerable

degree of divergence from our field strains and is genotype VII. The amino aidc substitutions of the F protein of field strains were found to be unique in this study and may be related to NDV's genetic evolution or a distinguishing feature from other subgenotype VII viruses, these variation could have an influence on vaccination strains that do not efficiently elicit host viral immunity, nonetheless, antigenically matched vaccines significantly limit viral shedding. The prevention of virulent NDV from infecting poultry involves intensive biosecurity as well as the proper administration of appropriate vaccinations [29, 30]. All NDV management plans must maintain strong biosecurity and create reasonable vaccinations based on locally circulating strains in Iraq.

Conclusion

The results of this study suggest that the recently circulating NDV strains in Iraq in 2020 include class II, a Genotype VII, pathotype very virulent and that the discovered strains have a large genetic variation from previously reported strains in Iraq, with a mean value of 84-95%, The NDV field strains (genotype VII) should be genetically matched to the vaccination strains used in Iraq to combat NDV. Also In endemic area, NDV vaccinations without biosecurity may fail to control the disease. To comprehend more about how virulent NDV strains circulate, extensive molecular epidemiology studies of NDVs with huge sample number are required.

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Author's Contribution

In the preparation of the manuscript, all authors contribute equally.

Conflict of interest.

"The authors declare that there are no conflicts of interest regarding the publication and/or funding of this manuscripts.

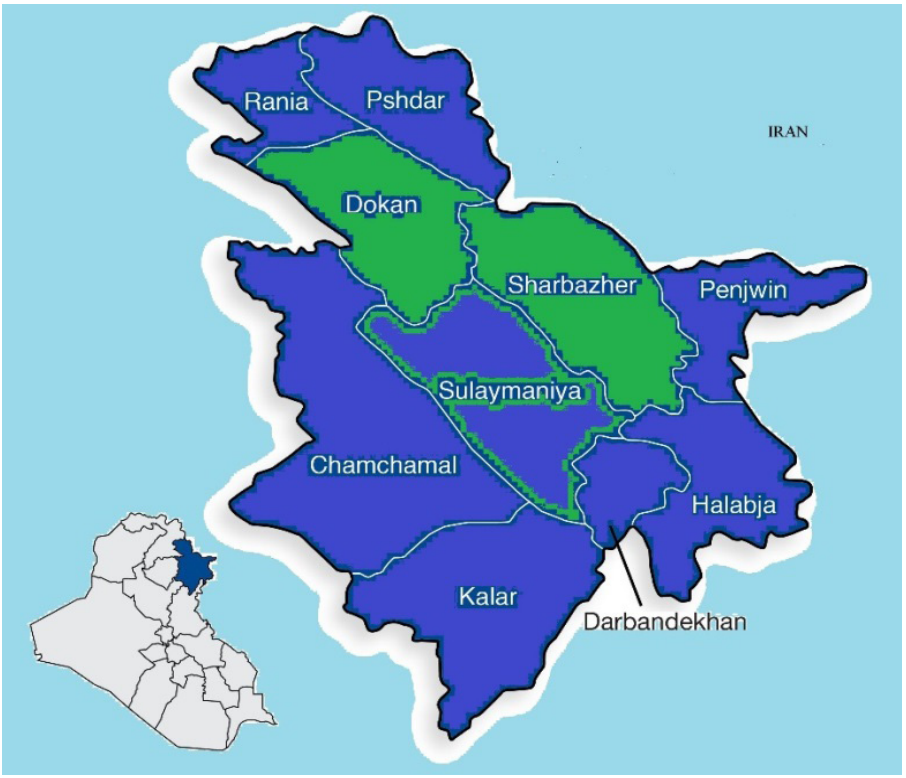

Fig. 1. District used for the current study (Dukan, and Sharbazher) for the geographic region (Green Zone) where samples were taken

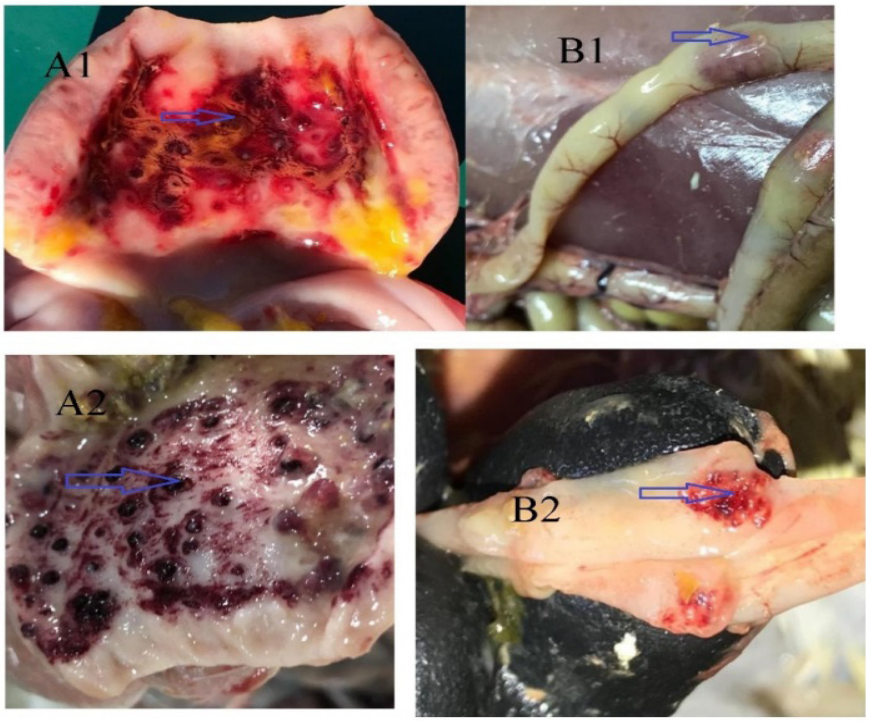

Fig. 2. Post mortem lesion natural broiler infection (25, and 30 days) from field Sulaimani/Iraq; (A1 and A2) sever congestion and hemorrhages in proventriculus; (B! and B2) sever congestion and hemorrhages in cecal tonsils.

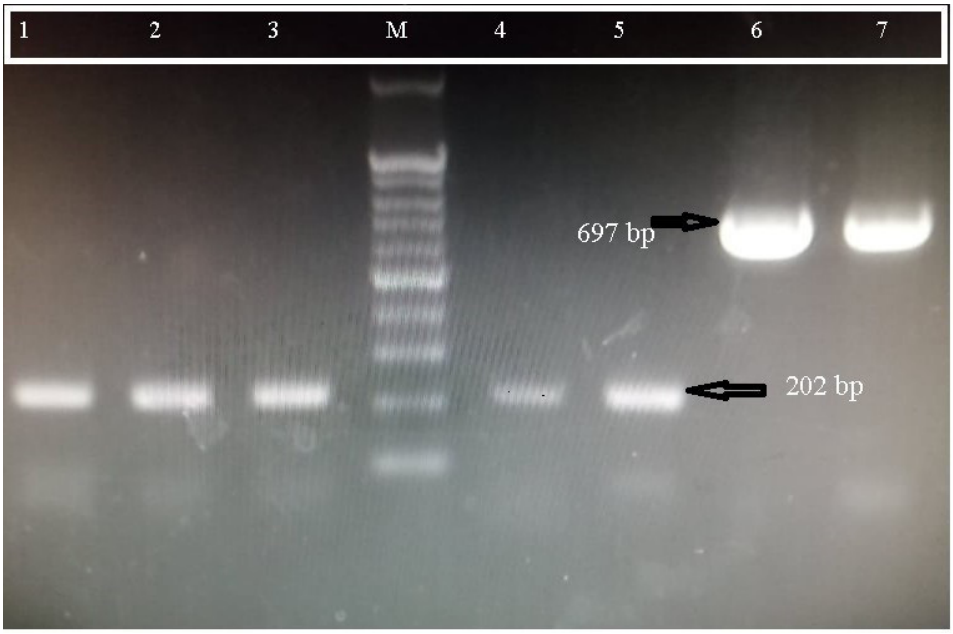

Fig. 3. Agarose gel amplification of the target region primer NDV-f/NDV-r (202bp), and primer M1055/ F508 (697 bp). Lane M: DNA marker, Lane 1-5 NDV positive (202 bp), Lane 6 and 7 positive for NDV (697 bp).

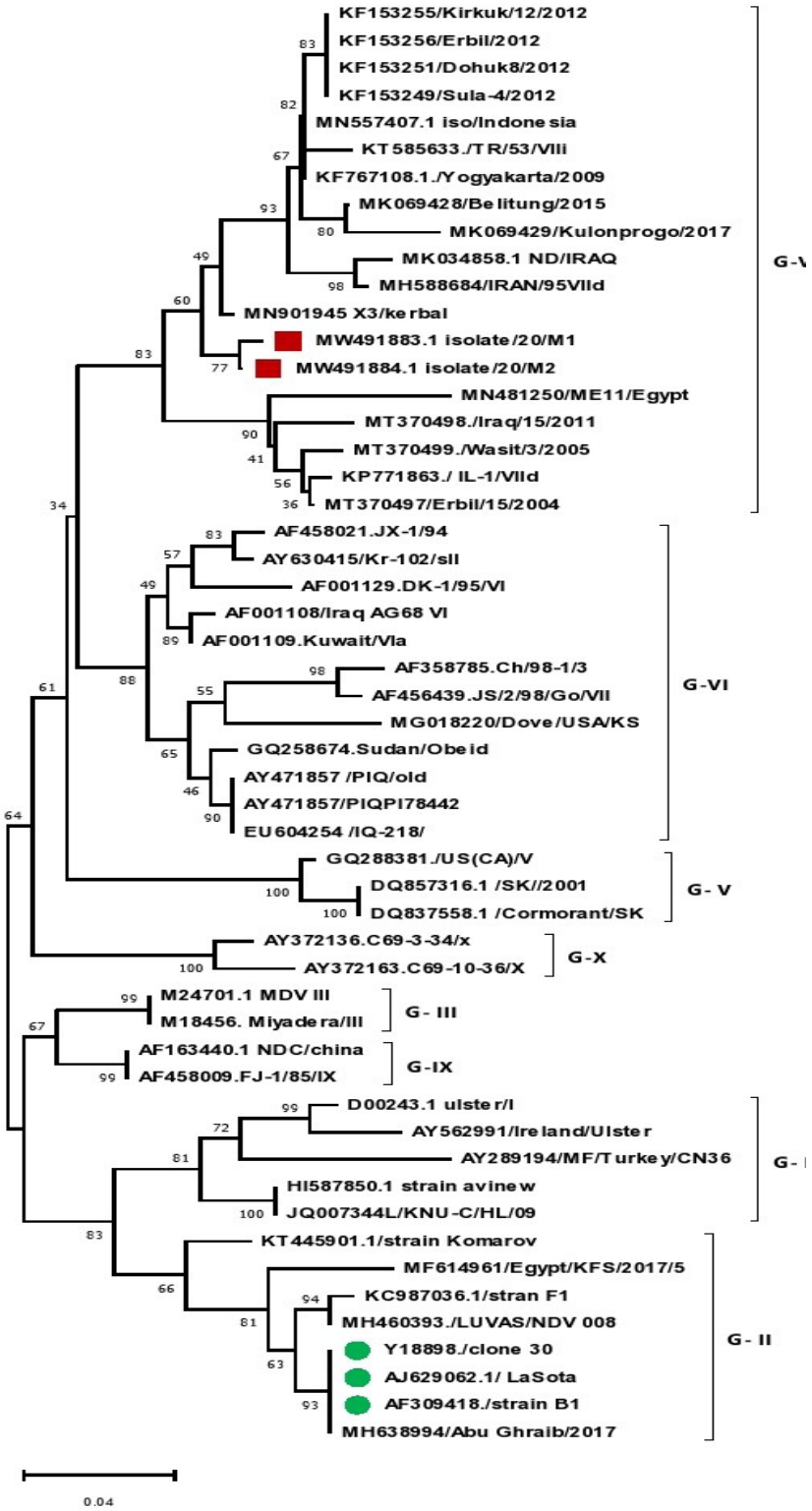

Fig. 4. Neighbor-joining method for tree constructed, phylogenetic study of field isolated vNDV strains of partial sequence (bold Red rectangle), from various genotype were include as a representative, (commercial vaccine strains bold Green circle). MEGA X was used to discover phylogenetic relationship through 1000-boostrap experiment utilizing the clustalW alignments algorithm

TABLE 1. Compares the sequences of the field virus with commercial vaccines strains and other countries.

Accession No.	Strain	DNA identities	a.a identities	Genotype
D00243.1.	ulster/I	86.36 - 85.71	86.36 - 85.71	I
Y18898.1.	clone_30	83.33 - 83.12	83.12 - 82.47	II
AF309418.1	strain_B1	83.12 - 82.90	81.82 - 81.17	II
AY562986.1	(Fl)44083	88.95 - 88.43	89.15 - 88.37	I
AY562991.1	Ireland/Ulster	84.32 - 83.80	80.62 - 79.84	I
M24696.1	Lasota	83.33 - 83.12	83.12 - 82.47	II
AF217084.1	strain_v4	86.12 - 86.63	85.27 - 86.05	I
HI587850.1	avinew	86.12 - 86.63	85.27 - 86.05	I
KT445901.1	Komarov	82.68 82.47	84.42 83.77	II
MN557407	Indonesia	95.89- 95.67	98.70 -98.05	VII
MT920211	Pakistan	95.67- 95.45	98.05 97.40	VII
KT585633.1	Turkey	94.71	96.21 95.45	VII
MH588684.1	Iran	95.02- 94.81	96.75-96.10	VII

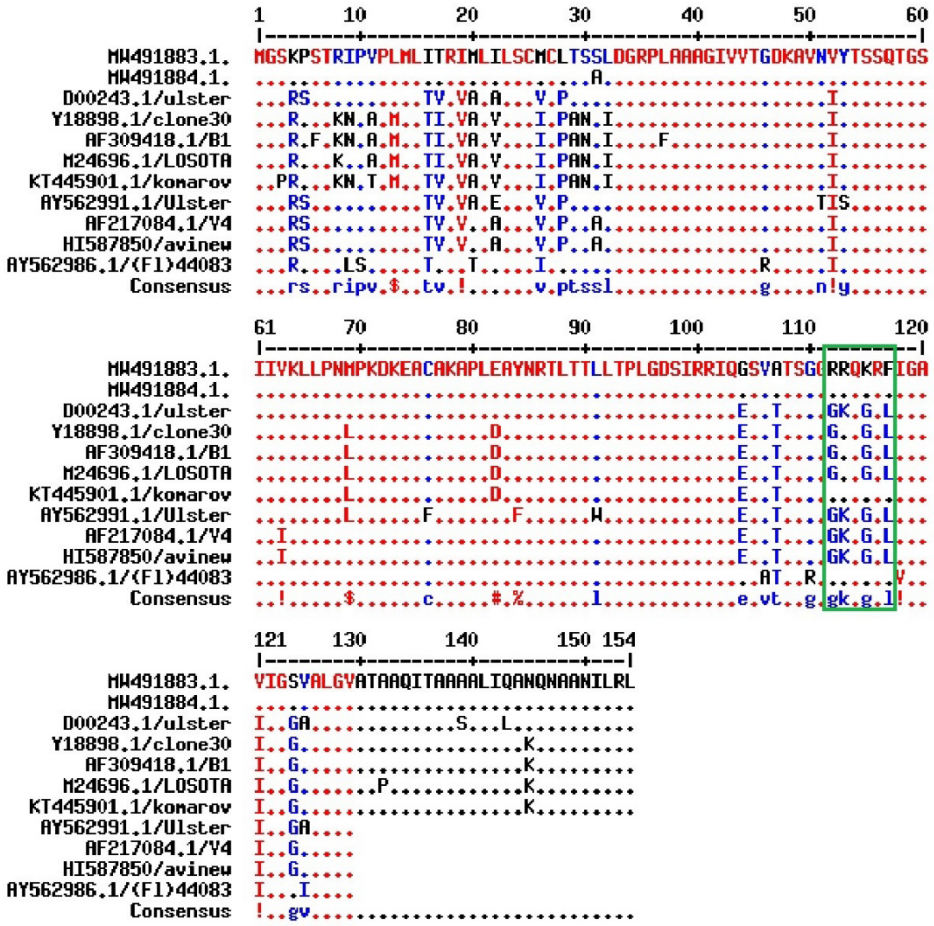

Fig. 5. Deduced amino acid alignment between field virus strains NDV of partial sequence F gene with commercial vaccines strains at position (1-154) including motif cleavage site (highlight in Green box).

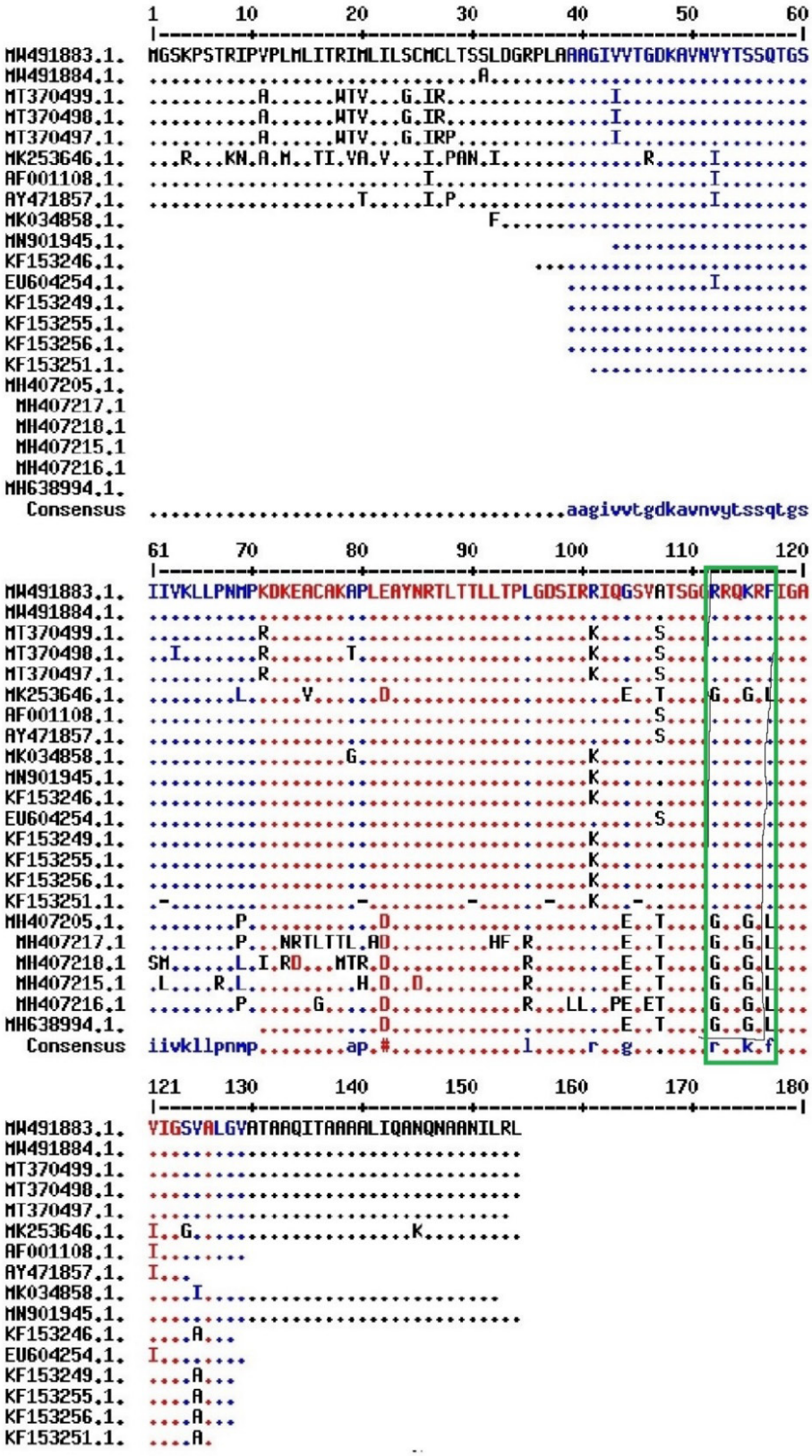

Fig. 6. Deduced amino acid alignment between field virus strains NDV of partial sequence F gene and other Iraqi strains at position (1-154) including motif (highlight in green box).

TABLE 2. Compares the sequences of the field virus to earlier Iraqi sequence NDV viruses.

Accession NO.	Strain/year	DNA identitie	a.a identities	Genotype
MH407205.1	Moh4/Iraq/2016	83.69 - 83.69	89.36	II
MN901945.1	X3/Kerbala/IQ/2019	97.63	99.11	VII
AY471857.1	PIQPI78442/1978	92.51 - 92.25	95.16 - 94.35	VI
EU604254.1	Q-218/78	92.03	96.70	VI
AF001108.1	Iraq AG6	92.80 - 92.54	96.90 - 96.12	VII
KF153246.1	Erbil/12VIR11/2012	96.44 - 96.80	97.85	VII
KF153249.1	Sulaymaniya/ 2012"	96.69 - 97.06	97.78	VII
KF153251.1	Dohuk/12VIR18/2012	94.96 - 95.35	94.05	VII
KF153255.1	Kirkuk/12VIR100/2012	96.69 - 97.06	97.78	VII
KF153256.1	Erbil/12VIR1-13/2012	96.69 - 97.06	97.78	VII
MH638994.1	Abu Ghraib/2017	83.98 - 83.43	86.44	II
MK034858.1	Iraq/2018	95.59	96.69	VII
MT370499.1	Iraq/2005	93.07 - 92.42	92.86 - 92.21	VII
MT370498.1	Iraq/15/2011	92.42 - 91.77	91.56 - 90.91	VII
MT370497.1	Erbil/15/2004	93.29 - 92.64	92.16 - 91.50	VII
MK253646.1.	AMHA1/Iraq/2012	82.59 - 82.34	81.82 - 81.17	II
MH407217.1	NVMoh16/Iraq/2016	76.95	77.66	II
MH407218.1	NVMoh17/Iraq/2016	79.43 - 79.08	79.79	II
MH407216.1	NVMoh15/Iraq/2016	80.50 - 80.14	80.85	II
MH407215.1	NVMoh14/Iraq/2016	81.91	84.04	II

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التنوع الجيني للنمط الجيني السابع، وفيروس مرض نيوكاسل الخبيث(NDV) ، الذي يحدده بروتين الاندماج في موقع الانقسام، وعدم التجانس مع سلالات اللقاحات التجارية

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كان الهدف من هذه الدراسة هو معرفة سلالة فايروس النيوكاسل الاكثر شيوعا المسؤلة عن تفشي الفيروس في مزارع الدجاج في محافضة السليمانية/ العراق. على الرغم من تنفيذ برنامج التطعيم الشامل لمزارع الدواجن، فقد انتشرت العدوى إلى دجاج التسمين التجاري. تم التمييز الجيني بين سلالات الفيروس والعينات المرضية باستخدام موقع انقسام البروتين الئيف الانصهار. تم تحديد العزلات على أنها تنتمي إلى الفئة الثانية ، النمط الجيني السابع، عن طريق التحليل الوراثي بناء على تسلسل جزئي لجين البروتين الئيف، على عكس سلالات اللقاح التجارية التي كانت تستخدم في كثير من الأحيان ، والتي كانت تنتمي إلى النمط الجيني الثاني من الفئة الثانية. هذا هو البحث الأول الذي يجد مثل هذه السلالات من فيروس نيوكاسل في مزارع الدجاج التجارية في محافظة السليمانية. لضمان الإدارة السليمة والاستجابة للسلالات الناشئة ، يجب تقييم بروتوكولات التطعيم الميدانية بانتظام ، أيضا لتقليل احتمالية أوبئة مرض نيوكاسل الدواجن ، يجب تنفيذ تدابير صارمة للأمن البيولوجي.

الكلمات الدالة: الجينات الاندماجية، شديدة الفوعة، التنميط الجيني، شجرة النشوء والتطور، منتشرة.