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Newcastle disease virus detection and characterization using molecular biology techniques

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Abstract
The identification of ND (Newcastle disease virus) by polymerase chain reaction examination completes the methodology of diagnosis of the disease, through remarkable sensitivity and speed in generating results. Also, the analyzing of the cleft sites of the fusion's gene (F gene), at the level of component nucleotides and subsequently their translation into specific encoded amino acids, provides important data on the pathogenicity degree of isolated virus strains. Thus, molecular biology techniques complement the diagnosis and characterization of Newcastle disease strains, being successfully used for herd surveillance. The study consisted of molecular analysis of (47) virus strains isolated between 2016 and 2022, of the vaccine strains used to combat the disease as well as of the pathogenic strain with the sign "*Craiova*" used to test the effectiveness of vaccination.

Keywords: Newcastle disease, PCR, cleavage site, molecular characterization, pathotyping

Introduction
The poultry paramyxovirus, which is another name for Newcastle disease, significantly contributes to the economic losses in the poultry industry in Iraq, due to the high morbidity and mortality indices recorded in the industrial and extensive breeding sector, the quarantines instituted in response to the disease's first outbreak and subsequent efforts to contain it [1]. The diagnosis of the disease must be rapid and accurate in order to generate a prompt response to the newly emerged situations; in this respect, molecular biology techniques complement virological methods of viral identification and characterization [2].
Poultry are susceptible to Newcastle disease virus (the Newcastle disease virus), a type 1 paramyxovirus that belongs to the Paramyxoviridae group. The genome is around 15 kilobases long and is made up of a single strand of RNA that is negative sense oriented. Polymerase, hemagglutinin, neuraminidase, fusion, matrix, phospho, and nucleo-capsid components are the six components of the viral genome [3, 6].
The pathogenicity of the virus relies on the specific amino-acid sequencing at the region of cleavages of the F Gene, as well as the capacity of cellular proteases to cleave the precursor. During the process of replication, viral particles are generated containing a glycoprotein precursor (F0), which needs to be divided into segments F1 and F2 in order for the virus to obtain infectious characteristics [4]. The process of post-translational cleavage is carried out by proteases present in the host organism's cells. It seems that the F0 molecules belonging to the velogenic/mesogenic category viruses can be cleaved by the host organism's ubiquitary proteases, so one can speak of a systemic viral dissemination, with damage to vital organs and the appearance of clinical signs of disease [5]. For lentogenic viruses, F0 cleavage is restricted by the presence of trypsin in host cells, so viral localization is only possible where it benefits from the existence of this enzyme (predominantly at gut level) [3].

Materials and Methods
The virus: The material under investigation was represented by 47 strains of NDV identified with reference positive serum, isolated in the period 2016-2022 from various disease outbreaks in the industrial and extensive sector (households). Also, the vaccine strain Ceva New L (Ceva Sante Animale), the antigen from the ND-TEST kit (Romvac Company), as well as the strain with the sign "*Craiova*" used for testing the effectiveness of vaccines (Virus Laboratory, Diyala veterinary hospital, Iraq) were subjected to investigations.

RNA extraction

The extraction was performed using the "Qiamp viral RNA Mini Kit – Qiagen" with 200 µl of supernatant sample and elution of RNA in a final volume of 50 µl, following the manufacturer's recommended methodology. Reverse Transcription Polymerase Chain Reaction (RT-PCR) reaction. Following the manufacturer-recommended protocol, we used the Qiagen 1 Step a real-time kit to perform both the RT and Polymerase chain reaction steps in a single reaction. The primer used were those recommended by International Reference Laboratory Padua, Italy, with the amplification of a fragment of the F gene that also encompasses the cleavage site.

Electrophoresis and purification of reaction products. For the visualization of the specific bands, electrophoresis was performed in agarose gel, with the identification of the fragments of interest based on the specific dimensions of 320 base pairs (Fig. 1). Next, amplicons undergo a purification process from agarose gel, using the 'MinElute Gel Extraction Kit-Qiagen' kit, according to the protocol recommended by the manufacturer.

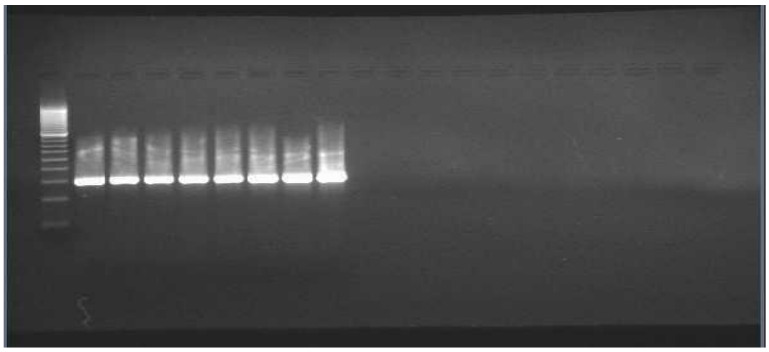

Fig 1: Electrophoresis of amplification products (Line 1 molecular marker 100 pb, lines 2-8 samples subjected to analysis, Line 9 positive control, lines 10 and 11 negative extraction and amplification witnesses).

Direct sequencing of amplification products. For the PCR reaction, the "BigDye Terminator Cycle Sequencing Kit-Applied Biosystems" was used, the protocol recommended by the manufacturer, with an alignment temperature of 58 ºC**.** The purification of the resulting amplicons was performed on purification colonies (Centri-Sep Columns-Princeton Separations), the protocol recommended by the manufacturer, followed by precipitation (pellet production) of nucleic acids with ethanol and their resuspension in denaturing agents (Hi-Di-Formamide Applied Bio-systems)**.** Capillaries electrophoresis were performed on the model 3130 - Applied Biosystems genetics Analyzers, using polyacrylamide as a separation Matrix (POP 4 - Applied Biosystems) [10].

Results and Discussions

The real time RT-PCR technique allows rapid identification of the viral antigen (the results are obtained in about 3 hours), a reduced risk of inter-contamination of samples (by lack of electrophoresis stage, performing in a single reaction of reverse transcription and amplification of the gene segment). The sensitivity of the technique was evaluated by comparing it with the conventional RT-PCR reaction, using a virus strain with a titer of 1:512 to the hemagglutination inhibition reaction, from which decimal serial dilutions were performed. From the comparison of the obtained results, the superiority of this method over the conventional one is noted, being able to identify the presence of viral RNA up to dilution 10-7 (Fig. 2).

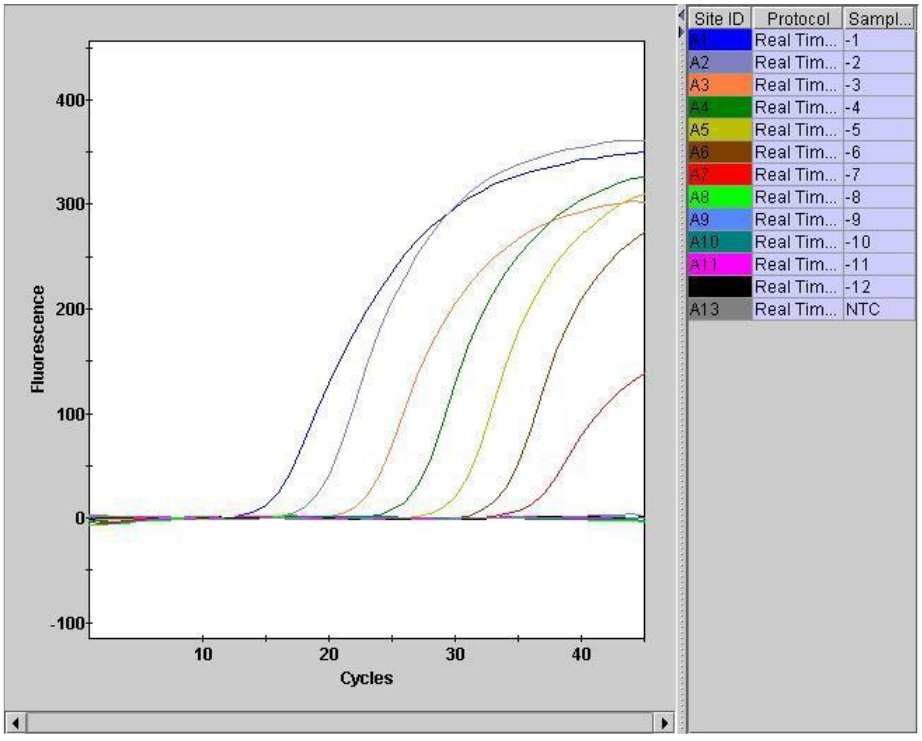

Fig 2: Results of real time RT-PCR test performed on decimal serial dilutions

The nucleotide sequences obtained were aligned using dedicated programs (Mega Software version 3.1, ClustalW Multiple Alignment), highlighting the cleavage site (Fig. 3) [5].

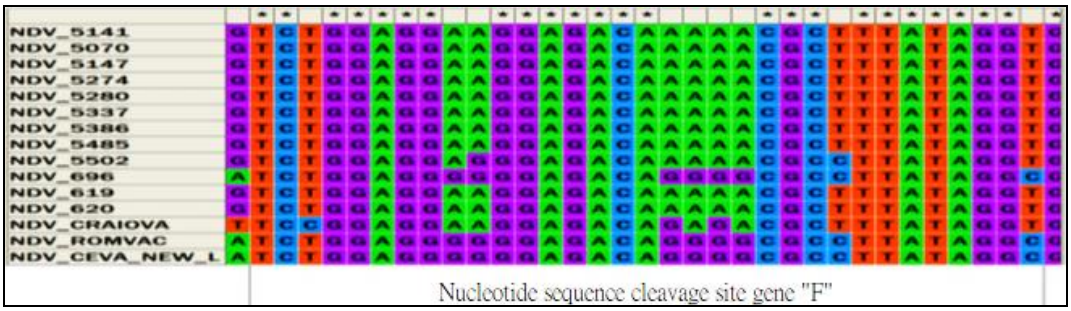

Fig 3: Cleavage site of the fusion protein gene (for image clarity, not all sequences obtained were introduced)

From the molecular point of view, the cleavage site of the F gene presents a nucleotide sequence made up mostly of adenine – A and guanine – G, which by post-translational phenotypic expression give rise to a specific amino acid sequence. In this sense, two necessary amino-acids at regions 112 and 113, and two more at regions 115 and 116, plus the amino acid phenylalanine (F) in position 117, causes a recognition of the site by the ubiquitous proteases, with systemic viral replication and belonging of the strain to the mesogenic / velogenic category. The amino acids found in positions 112 and 113, respectively 115 and 116 are represented in this case by lysine (K) and/or arginine (R) in theoretically possible combinations. If only arginine (R) is present in the two positions, it can be said that the isolate belongs to the velogenic category [11]. In contrast, the lentogenic strains present in positions 112 and 113, respectively 115 and 116, in addition to the aforementioned amino acids, also the amino acid glycine (G) in theoretically possible combinations. Moreover, in position 117, instead of phenylalanine, leucine (L) is present (Fig. 4).

Thus, from the analysis of the results it can be seen that, for most strains isolated from the field, the amino acid sequence was 109SGGRRQKRFIG119, being the strains belonging to the mesogen/velogen category. On the contrary, for some isolates (NDV 5502, 696), the amino acid sequence was 109SGGGRQG(K)RLIG119, being isolated strains from birds vaccinated against pseudopesta and thus with an amino acid sequence compatible with the lentogen category, demonstrating the vaccine origin of the isolated strains.

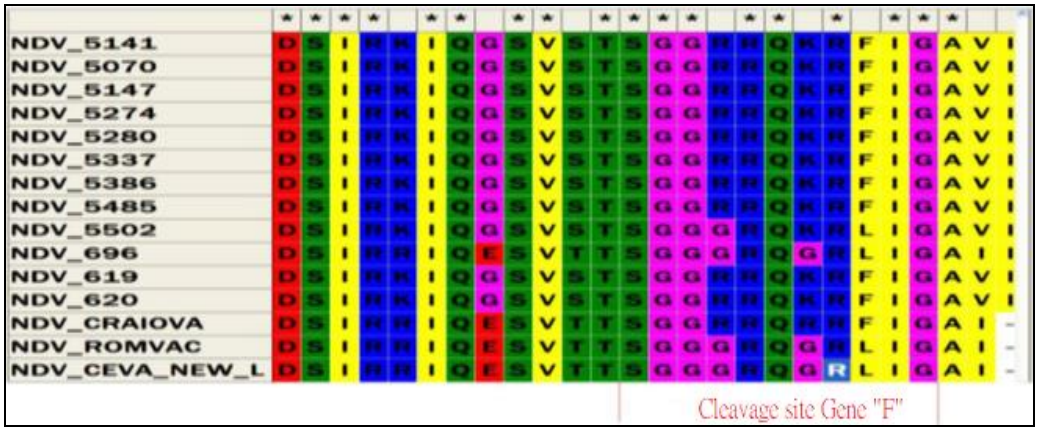

Fig 4: Amino acid sequence resulting from nucleotide translation at cleavage site level (for image clarity, not all obtained sequences were introduced).

Regarding the strain with the designation" Craiova", the amino acid sequence 109SGGRRQRRFIG119 places the viral isolate in the category of strains with the highest degree of virulence (Velogenic category). On the contrary, for the romvac antigen, but also in the case of the CEVA vaccine strain, the amino acid sequence 109SGGGRQGRLIG119, compatible with the lentogen category, certifies their use for the designated purposes [12].

The environment variables, such secondary immuno-compromising disorders, may be the reason why the Newcastle disease virus epidemics happen in inoculated individuals. The proposed explanation for the occurrence of NDV outbreaks in vaccinated populations may be attributed to environmental factors, such as secondary immunocompromising diseases. diminishing the effectiveness of vaccines [13]. Furthermore, the lack of collective immunity in the flock, in addition to insufficient vaccination protocols, may result in inefficient immunization and the recurrence of NDV occurrences [14]. A number of studies have looked into ways to treat the disease by developing antiviral drugs, such as a substance derived from the leaves of the natural plant *Olea's europaea* [15] limonin and arctigenin, two phyto-chemicals, have been recognized [16, 17].

The Abu-Ghraib_2017 isolate was deemed exceedingly hazardous by all pathogenicity indicators as evidenced by its MDT score of less than 50 hours. The ICPI and IVPI values that were detected were 1.96 and 2.56, correspondingly. The values mentioned serve as indicators of the unique characteristics that are attributed to velogenic (the Newcastle disease virus) isolates, in accordance with the guidelines established by the World Organisation for Animal Health (OIE) [18].

Finding out that the molecular study of the Abu-Ghraib, 2017 isolation has yielded useful insights is a really interesting and

fascinating development. The presence of an avirulent (lentogenic) fusion protein that motif (GRQGRL) was observed in this specific strain, which has been demonstrated to be aggressive through both clinically and laboratory testing. In a surprise turn of events, this pattern coincides with the arrangement of the hyper-variable area of the combined protein. Through the course of the investigation, the investigators investigated the molecular properties of the incomplete combining protein that was discovered in the Malaysian virulent isolates that were taken from clinically ill individuals. The researchers made the discovery that a specific velogenic strain, with the number of its accession MB016/07, possessed the GRQGRL pattern, which is a widely detected non-virulent pattern [19].

In addition, what we discovered align with the results obtained from China [20], a situation in which it was found that the majority of genotyping 2 strains that had the lentogenic pattern 112G-R-Q-G-R-L117 displayed aggressiveness. They reviewed the strong resemblance that was detected among the strains of genotypes 2 and the original isolates LaSota, which is in perfect alignment with the study that we have really conducted. In order to differentiate among isolates of (The Newcastle Disease viruses) that are low virulence and those that are aggressive, it was hypo-thesised that the examination of F gene cleaved location patterns alone might not be adequate. Therefore, the discovery of aggressiveness can be dependent on the operation of additional genes that are present in the genetic material of the virus. Hence, in order to properly characterise NDV, it is necessary to combine a number of different biological-examinations and investigations of the F-protein.

The F-gene reference is a polypeptide consisting of (553) protein that requires activation by dividing via the host cell's proteases, resulting in the formation of F1 and F2 subunits. The arrangement of amino-acid sequences at the proteolytic breakdown site is what determines the substrate specificity as well as an infectiousness of the virus. Through the modification of the protein configuration at the F-gene cleavage site, the virulence of a lentogenic NDV was elevated to a mesogenic phenotype. This was accomplished by converting the mono-basic configuration to the poly-basic configuration [21].

There is additional glyco-protein that is also an exterior glyco-protein, and the gene called F that is produced by the NDV is one of them. The procedure of the virus invading the host cell is facilitated by this method, which involves the fusion of the viral outer layer with the plasma membrane. Beginning with the production of a preliminary amino acids that is composed of (553) proteins, the gene known as F is created. It is necessary for it to go through the process of cleavage into two subunits, (F1 and F2), which are linked together by di-sulfide bonds for the purpose for it to get active [22]. The (proteases) that are present in the host-cell have an effect on the breakdown of proteo-lytic procedure [23], which differ in the way that they identify the protein chains among mono-basic and poly-basic patterns that they have identified. Therefore, the specific sequence of protein at the site of proteo-lytic cleavage is what determines the choice of the base, which in turn has an effect on the virus's capacity to replicate [24, 25, 26].

The modification of a single basic amino acid sequence at the area of cleavage of the F protein to a multiple basic motif resulted in an enhancement of the pathogenicity of a mildly virulent Newcastle Disease Virus (NDV) to a moderately virulent phenotype [27, 28], On the other hand, it cannot reach the condition of having a viral infection that is entirely aggressive (velogenic). To add insult to injury, PPMV-1 isolates that are hazardous to doves may have a poly-basic breakage place, yet they are not causing illness in chickens [29]. As a result, the poly-basic F-gene cleavage region can't, on its own, determine whether or not the NDV is harmful.

This research paper provides information regarding the use of deformed oligo-nucleotide primer for the purpose of amplification of DNA sequences obtained from genomic of several isolates of the NDV. The same primer were used to immediately sequenced the products of amplifying using an automated nucleotide sequencing after the reverse transcription-polymerase chain reaction (RT-PCR) sequence was performed. Using phylogenetic methods and connecting with the nucleotide or predicted protein sequences, it can be achieved to distinguish ineffective lentogenic strains of the NDV from the most aggressive (mesogenic) and (velogenic) strains. This may be done in a uniform way. Both the fusing amino acids genome sequence and the structure of the matrix proteins genetic real-time PCR outcome sequencing both produced results that were equivalent to one another. As a consequence of this, it is possible that these processes are going to end in a reduction in the usage of animals that are alive for the purpose of determining the infectiousness of infectious isolates caused by the NDV. The alignment of the hierarchy of relationships, which have been verified by the DNA sequences of the real-time PCR products, with connections that have been reported in the past for the two entire the protein fusion gene and the fusion protein genome is of the utmost importance [30], in addition to the hem-agglutinin and neur-aminidase genes produced by a number of strains of the Newcastle disease virus [31].

A genotype One viruses of chicken paramyxo-virus is responsible for the Newcastle virus, which is a disease that almost always results in death in poultry. Both pathogenic (velogenic) isolates and low pathogenic (lentogenic) isolates are distinct forms of Newcastle Disease Viruses, which can be categorised into two distinct categories. There is a correlation between the biological characteristics of the viral F glyco-protein and the infectious profile of the the Newcastle disease virus virus [32]. The F-proteins is originally synthesised as a precursor with no activity gene known as F0 during the procedure of virus multiplication. This occurs prior to the production of the F-protein. In order for the F protein to become active, it must undergo post-translational cleavage by host proteases, resulting in the production of active F1 and F2 proteins. Without these active proteins, union between the host cell and viral membranes cannot take place, rendering the virus non-infectious [31]. The susceptibility to cleavage and variation among various the Newcastle disease virus isolates is determined by the structure at the F0 division site. The C-terminal of the F2-protein in virulent strains of the Newcastle disease virus contains a minimum of 3-arginine or lysine-residues between residues (113 and 116). These residues are located between one another. Additionally, there is a phenylalanine-F particles located at site (117) in the the N terminal of the F1-protein. This motif seems to make F0 susceptible to cleavage by furin, a widely distributed protease, allowing virus infection to propagate within the host [33, 34]. On the other hand, low pathogenic the Newcastle disease virus isolates possess just 2 individual basic amino acids at positions 113 and 116, together with leucine at position 117. The F0 protein from these viral is specifically digested by proteases that are comparable to trypsin. Only a select few organs contain these proteases; for example, the cells of the

epithelium that make up the systems of respiration and digestion are their only known locations. Therefore, the infection that is brought on by these viral agents continues to be confined. It is possible to differentiate between extremely virulent and less highly infectious strains of the Newcastle disease virus by reading the nucleotides that are located at the location of the break of the F-protein [35].

Conclusions

1. The real time RT-PCR technique can be successfully used in the disease surveillance strategy, due to the advantages generated by sensitivity (both over virological methods and over conventional RT-PCR), speed in generating results, ease of obtaining biological material under investigation (especially for wild birds - fecal samples, cloacal swabs, etc.).
2. Molecular analysis of the cleavage site and consecutive classification of the viral isolate in one of the three existing categories (Lentogenic, Mesogenic/Velogenic and Velogenic) is one of the most accurate techniques of viral characterization in order to establish pathogenicity (pathotyping). Thus, the method can be successfully applied for confirmation, the results are obtained in a shorter time compared to Virological methods; also, in cases where, for various reasons, the isolation and characterization of the virus cannot be achieved, being possible only the amplification of specific nucleic acids, the technique is a viable alternative to achieve these desiderata.

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