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**The Inflammatory Biomarkers Profile of Broiler Chickens
Infected with Newcastle Disease Virus Recently Circulated
in Thi-Qar Province**

**A Thesis Submitted to Council of Veterinary Medicine College,
University of Thi-Qar in Partial Fulfillment of the Requirements for
Master Degree in the Veterinary Medicine / Microbiology**

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2024 A.D

1446 A.H

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Acknowledgment

First of all, I would like to express deep thank to the lord of the universe Allah for the success in accomplishing this work. Then, I would like to thank and appreciate the Dean of Veterinary medicine College and the Head of the Microbiology Department. Also, I am grateful for my supervisors, **Dr. Hakeem Jawad Kadhim** and **Dr. Safaa Hussein Ali** to support me for performing this research.

My thanks are written for the laboratory of the undergraduate and postgraduate studies of the mentioned faculty. Additionally, I thank **Dr. Hatam Abdulkareem Jabr** / Sumer University, **Dr. Moatamad Razzaq Kadhum**/ Aldawayah Veterinary Hospital and **Dr. Falah Hassan Abd** / Alshatrah Veterinary Hospital for their assistance in sample collecting and advice providing.

Finally, I would like to thank all the crew of the Central Veterinarian Laboratory in Baghdad especially, **Dr. Yusra Jassim Abbas** and **Dr. Salma Talib Alhassani** for the instructions and examining the samples.

Abbas

Dedication

-To the one who illuminated the path of darkness, the nation's Mustafa and its guide, Muhammad (God prays on him)

-To all the martyrs of Iraq

-To the one who exerted the effort of years and forged the ladders of the highest days to ascend to the heights of life....my father

-To whom God Almighty has allocated Paradise under her feet... ...my mother...

-To the one in which God Almighty said, "And we created for you mates from among yourselves." My wife

-To the innocent eyes that look at me with love..... my daughter

-To those whose hearts beat for me with love and fear for me..... my brothers and sisters

-To the candles that lit the path of knowledge for me.... my teachers

I dedicate this work

Abbas

Summary

The current study was designed to identify endemic serotype of Newcastle disease virus (NDV) from flocks suffering from severe infections that are characterized by high mortality, respiratory, digestive, and nervous signs within Thi-Qar Province.

Depending on the case history, clinical signs, and necropsy, 83 samples (suspected) and 40 samples as (control) were collected from about 15 flocks of broilers between the ages of 20-35 days during the period from October 2023 to February 2024.

At the beginning of the experiment, a rapid NDV test was utilized to confirm the infection. Blood, liver, trachea, lung, and cecum were collected in a 1.5 ml tube containing about 1ml of Trizol for RNA extraction. Also, serum was isolated from blood samples and frozen at (-20 °C). All other samples were snap frozen in liquid nitrogen (-196 °C).

The positive samples detected by the NDV rapid test were assigned to the molecular approach. Therefore, total RNA was extracted, purified, and quantified using a nanodrop spectrophotometer. Thereafter, RNA was converted into complement DNA (cDNA) that was subjected to real-time polymerase chain reaction (rt-PCR) techniques to detect nucleic acid NDV in the samples. Reverse transcription-polymerase chain reaction (RT-PCR) was utilized to study the gene expression of inflammatory biomarkers. Also, conventional PCR and nucleic acid sequencing were performed to identify genetic variations for the F gene (356 bp) and the HN gene (416 bp).

In the current study, several bio-inflammatory markers have been tested in the positive samples by RT-qPCR to observe the inflammatory response induced by NDV in infected birds compared with healthy birds. Specifically, C -reactive protein (CRP) gene expression increased significantly in birds after two days (48 hrs) of infection and documented about two fold changes. In contrast, interleukin 6 (IL-6), matrix metalloproteinase 9 (MMP-9), interleukin 1 beta (IL-1 β), and interferon gamma (Inf- γ ,) mRNA expression were upregulated after 24 hours of infection and displayed 3-4 fold changes in mRNA expression compared with their expression in non-infected birds. Therefore, the high temperature resulted from viral infections, and severe inflammation occurred because of the same infection with the ND might be due to the high immune response. Furthermore, a molecular approach, PCR, was applied to identify genetic variations in the ND virus to explain ND outbreaks in Thi-Qar Province despite regular and intensive immunization program.

Nucleotide sequence data for F and HN genes were shown at 88.27% and 89.44% similarity with data in the NCBI, respectively. In conclusion, data explained the recurrent NDV infections in Thi-Qar Province despite the intensive immunization program causing high mortality rates. Therefore, it is required to use a vaccine that has high similarity with NDV strains endemic in the Province.

List of Contents

Subject	Page number
Summary	I
List of contents	III
List of tables	VI
List of figures	VII
List of abbreviations	VIII

Chapter One- Introduction

Introduction.....	1
Aim of study.....	3

Chapter Two- Literature Review

Introduction and historical overview for Newcastle disease	4
Taxonomy and structure of Newcastle disease virus.....	6
Viral proteins.....	7
Biological properties of paramyxoviruses	9
Hemagglutination and neuraminidase activity	9
Antigenicity.....	10
Virus entry.....	10
Replication.....	10
Assembly and release.....	12
Infection mechanism.....	12
Resistance, virulence, and pathogenicity	14
Pathology.....	15

Genetic diversity and geographic distribution.....	19
Host species.....	22
Mode of dissemination.....	23
Clinical signs and lesions.....	23
Diagnosis.....	25
Pathogenicity testing.....	27
Control.....	28
Vaccine and vaccination.....	30
Types of vaccine.....	32
Live vaccine.....	32
Inactivated vaccine.....	32
Recombinant vaccine.....	33
DNA vaccine.....	33
Vaccination program.....	34
Inflammatory response induced by Newcastle disease virus.....	35

Chapter Three - Materials and Methods

Materials.....	39
Instruments and equipment.....	39
Chemicals, biological materials, and kits.....	40
Study design and collection of the samples.....	41
Diagnosis.....	43
Primary (suspected) diagnosis.....	43
Immunological diagnosis.....	43
NDV rapid kit.....	43
Molecular diagnosis.....	44

Reverse transcription–quantitative polymerase chain reaction for inflammatory markers using	44
RNA extraction.....	44
Reverse transcription (cDNA synthesis).....	47
Quantitative polymerase chain reaction (qPCR).....	47
Conventional polymerase chain reaction for HN and F gene.....	49
Viral RNA extraction from VTM.....	49
cDNA synthesis (using Superscript® III reverse transcriptase).....	50
PCR condition (Using Taq PCR Master Mix Kit (2x)).....	50
Agarose gel electrophoresis of DNA.....	51
Primers.....	52
DNA sequencing method.....	54
Statistical analysis.....	54

Chapter four - Results

Sampling and Initial diagnosis.....	55
Clinical findings.....	56
Clinical signs.....	56
Gross lesions in post mortem examination.....	57
Immunological detection.....	59
NDV Immunochromatographic rapid assay.....	59
Molecular detection of ND virus in the samples.....	59
Gene expression data of inflammatory biomarkers.....	61
DNA sequencing of selected region of gene F and HN of NDV.....	62
FASTA files isolates based on the sequences of F gene	62

Chapter five - Discussion

ND clinical manifestations and post mortem lesions.....	64
ND virus changes mRNA levels of inflammatory markers.....	66
Molecular changes in the ND virus.....	69
Conclusion.....	72
Recommendations... ..	73
References	74

List of Tables

Table	Page Number
Table (3-1).....	39
Table (3-2).....	40
Table (3-3).....	48
Table (3-4).....	48
Table (3-5).....	51
Table (3-6)	51
Table (3-7)	53
Table (4-1).....	55

List of figures

Figure	Page number
Figure (2.1).....	8
Figure (3.1).....	42
Figure (4.1).....	56
Figure (4.2).....	58
Figure (4.3).....	59
Figure (4.4).....	60
Figure (4.5).....	61
Figure (4-6).....	63
Figure (4-7).....	63
Figure (4-8).....	61

List of Abbreviations

Abbreviation	Meaning
AAvV-1.....	Avian avulavirus 1
AIV	Avian influenza virus
AOAV-1	Avian orthoavulavirus
APMV-1	Avian paramyxovirus 1
Bp.....	Base pair
CRP	C- reactive protein
C°	Celsius
CNS	Central nervous system
cDNA	Complement deoxyribonucleic acid
Ct.....	Cycle threshold
ELISA.....	Enzyme-linked immunosorbent assay
<i>E. coli</i>	<i>Escherichia coli</i>
et al.....	et alia (and others)
F.....	Fusion protein
HA.....	Hemagglutination
HI.....	hemagglutination inhibition
HN.....	Hemagglutinin-neuraminidase
H.....	Hour
IBV.....	Infectious bronchitis virus
IBDV.....	infectious bursal disease virus

ILTV.....	Infectious laryngotracheitis virus
Inf- γ	Interferon Gamma
IL-1 β	Interleukin-1 Beta
IL-6.....	Interleukin-6
ICPI	Intracerebral pathogenicity index
IVPI.....	Intravenous pathogenicity index
KD.....	Kilo Daltons
L.....	Large protein
LCR.....	Ligase chain reaction
MHC.....	Major histocompatibility complex
MDV.....	Marek's disease virus
MMP9.....	Matrix metalloproteinase 9
M.....	Matrix protein
mRNA.....	Messenger ribonucleic acid
μ L.....	Micro liter
Ml.....	Milliliter
Min.....	Minute
MABs.....	Monoclonal antibodies
Nm.....	Nano meter
NCBI.....	National center for biotechnology information
NDV.....	Newcastle disease virus
NP.....	nucleocapsid protein
P.....	Phosphoprotein

PPMV-1..... Pigeon paramyxovirus-1
 PM.....Post mortem
 PH..... Potential of hydrogen
 qPCR..... Quantitative PCR
 rt-PCR..... Real-time polymerase chain reaction
 RT-LAMP... Real-time reverse transcription isothermal loop amplification
 RBCs..... Red blood cells
 RT-PCR..... Reverse transcription-polymerase chain reaction
 RNA..... Ribonucleic acid
 Rpm..... Round per minute
 MSE..... Standard error of mean
 T cell.....Thymus cell
 TNF..... Tumor necrosis factor
 UV..... Ultra violet

Chapter One

Introduction

1.1. Introduction

Food security is a one of the most essential concerns for all of the world's people, especially in times of increasing food shortages. Most countries are working hard to enhance the food security situation so that food is accessible to all at low costs. The problem of protein shortage is exacerbated by an increase in population and the low per capita animal protein necessary for the body, as well as the problem of fodder and the difficulty of its availability, which makes the reliance on red meat as a source to provide the needs of the population of animal protein difficult.

This transferred attention to find alternatives available at a cheaper price of proteins for population needs. Poultry can be the fastest and most optimal solution, as poultry products, such as meat and eggs are among the fastest and cheapest sources for their production of animal proteins due to the high conversion efficiency (Mejlea, 2002; Al-Deeb, 2004).

Poultry breeding is considered one of the main sources of the agricultural economy because of its resources and products (meat and eggs), which hummans cannot abandon because there is no suitable alternative to replace them (Saleh *et al.*, 1984).

Many etiological agents have been isolated from farmed stock, ranging from bacteria, such as acute coliform bacillary, chronic tuberculosis, ecto- and endo-parasites, and fungal pathogens to at least eleven virus species that can be transmitted either horizontally, vertically, or both, such as the avian influenza virus (AIV), infectious bronchitis virus (IBV), Marek's disease virus (MDV), infectious laryngotracheitis virus (ILTV), infectious bursal disease virus (IBDV), and Newcastle disease virus (NDV) (Sid *et al.*, 2015).

Newcastle disease (ND) is caused by an infection of poultry species with virulent strains of Newcastle disease virus (NDV), is known as avian paramyxovirus 1 (APMV-1). The NDV may cause acute, virulent, and highly contagious diseases in poultry, characterized by damage to the digestive and respiratory tract besides central nervous system (Butt *et al.*, 2019). Furthermore, it has been stated that ND constrains the development of the poultry industry due to the high morbidity and mortality rates caused by ND. Additionally, it has been at least four major outbreaks of ND around the world, which have caused great harm to international trade. Each epidemic had a specific NDV genotype (Diel *et al.*, 2012).

Despite the wide use of ND vaccination programs in the poultry industry, different wild strains of the immunized flock are still isolated (Dimitrov *et al.*, 2021). Simultaneously, the emergence of atypical clinical symptoms and new strains have posed many difficulties in the diagnosis, prevention, and control of the disease. With the development of the global livestock and poultry industry and increasing in world trade, the prevention of ND is an important. Early detection via rapid, sensitive, and accurate methods is required for reducing the disease spread (Liang *et al.*, 2021).

1.2. Aim of study

Despite the intensive immunization program of ND in Iraq, ND is still the main danger for the poultry industry in the country. Therefore, the molecular identification of the endemic serotypes in each Province becomes an essential matter. This study is designed to identify the NDV in broilers within Thi-Qar Province, the following objectives will be addressed:

1. Determination of ND-positive cases based on clinical signs, postmortem lesions, and the NDV rapid test.
2. Gene expression changes in inflammatory biomarkers, such as CRP, IL-6, INF- γ , MMP-9, and IL-B using reverse transcription-qPCR.
3. Molecular diagnosis of NDV (Matrix gene) using RT-PCR.
4. Molecular diagnosis of the fusion (F) and haemagglutination neuraminidase (HN) genes by using conventional PCR.
5. Sequences and phylogenic tree for F gene of local strain isolates in Thi-Qar Province.

Chapter Two

Literature Review

2. Literature review

2.1. Introduction and historical overview for Newcastle disease

Many different types of birds are susceptible to Newcastle disease (ND). ND is a highly contagious and sometimes fatal illness which infects chickens, turkeys, and others. Depending on disease pathogenicity; ND can be classified as extremely pathogenic (velogenic), moderate (mesogenic), or mild (lentogenic)

The virus is known as the ND virus (NDV) and infects poultry and wild birds worldwide. The virus targets the respiratory, gastrointestinal, and central nervous systems of many different types of birds (Yune and Abdela, 2017). There are two categories (class I and class II) of velogenic strains of NDV responsible for virus global distribution. Class I contains almost all of the low-virulence strains collected from wild ducks across the globe. Class II strains including both low- and high-pathogenic strains which were isolated from domesticated and wild birds (Birhan *et al.*, 2019).

In 1926, ND was first reported in Indonesia (Amoia *et al.*, 2021). In 1927, Doyle isolated the virus in Newcastle, England, and named it NDV (Triosanti *et al.*, 2018). Then, numerous outbreaks of different avian species associated with virulent NDV were reported in North America (United States of America, Canada, and Costa Rica), Europe (France, Italy, England, Scotland, Spain, and Russia), Africa (Kenya), Asia (China, Korea, India, Japan, Sri Lanka, Kazakhstan, the Philippines, and Saudi Arabia), and Australia (Rahman *et al.*, 2018).

There are four ND pandemics worldwide. Since 1926, and each pandemic is caused by a different genotype of NDV. The first global

pandemic, which began simultaneously in Southeast Asia and Europe from 1920 to 1960, took approximately 30 years to become fully established. It was caused by NDV of the three genotypes of genes II, III, and IV (Wang *et al.*, 2015). The main infection targets are chickens, waterfowl, and birds, with almost no disease showing certain regional distribution differences (Bello *et al.*, 2018).

The second pandemic was from 1960 to 1970 and may have originated in the Middle East due to the increased commercialization of the poultry industry globally as well as the enhanced international trade of parrots. This was mainly caused by the V and VI genotypes of NDV, which endanger ornamental birds and caged birds (Pearson and McCann, 1975).

The third pandemic, believed to be caused by genotype VI isolates from late 1970 to 1980, occurred among racing pigeons but then spread all over the world and became difficult to control due to the relative lack of absolute control in racing pigeons' husbandry (Lumeij and Stam, 1985).

Finally, the fourth pandemic, which is currently ongoing, was believed to have started in the late 1980s and has been related to genotypes VII, V, VI, and VIII. It has caused economic losses in the poultry industry in many countries in Southeast Asia, the Middle East, Europe, Africa, and the Americas (Tan *et al.*, 2010).

2.2. Taxonomy and structure of Newcastle disease virus

Newcastle disease is caused by virulent strains of Avian orthoavulavirus1 (AOAV-1), formerly known as Avian avulavirus 1 (AAvV-1) and commonly known as Avian paramyxovirus 1 (APMV-1) or NDV. Newcastle disease virus belongs to the genus Orthoavulavirus, subfamily Avulavirinae, family Paramyxoviridae, and order Mononegavirales. Isolated paramyxoviruses are classified into 21 serotypes, designated APMV-1 to APMV-21. Each virus belongs to a viral species that is dispersed among the genera metaavulavirus, orthoavulavirus, and paraavulavirus (Amarasinghe *et al.*, 2019; Dimitrov *et al.*, 2019).

NDV is a pleomorphic, enveloped virus sized approximately 200–300 nm in diameter. Its envelope is derived from the plasma membrane of the infected cell, with an outer face where 8–12 nm-long spicules are inserted, corresponding to the HN glycoprotein and the F protein. The inner side of the envelope is lined with matrix protein. The viral genome is a non-segmented, single-stranded RNA of negative polarity. The RNA genome of NDV has a molecular weight of 5.2 to 5.7 x 10⁶ Daltons. Genome sizes vary between 15186 and 15198 nucleotides (Ali *et al.*, 2022).

The nucleoprotein combines with the RNA to form a tubular nucleocapsid with a diameter of 18 nm, and the nucleoprotein, phosphoprotein, and large protein are tightly bound to the genomic RNA (Mao *et al.*, 2022).

2.3. Viral proteins

The NDV genome consists of six genes, encoding six different proteins. The genes arranged in order of 3'-NP-P-M-F-HN-L-5' encode for nucleoprotein, phosphoprotein, matrix protein, fusion protein, hemagglutinin-neuraminidase protein, and large polymerase protein, respectively (Phale, 2018; Nurziah *et al.*, 2022).

The nucleoprotein has a length of 489 amino acids and a molecular weight of 55 kilodaltons (kD). It surrounds the genomic RNA and protects it from nucleases, while phosphoprotein is made up of 395 amino acids and forms multiple bands with a molecular weight ranging from 50 to 55 kD, which is essential in viral replication and transcription. Matrix protein (M) is a non-glycosylated protein associated with the inner surface of the envelope. It consists of 364 amino acids and has a molecular weight of about 40 kD. It plays an important role in viral assembly and envelope stabilization. Glycoprotein (F) consists of 553 amino acids and fuses the viral envelope with the membrane of the target cell.

Glycoprotein (HN) is a 577 amino acid-long polypeptide with a molecular weight of 74 kD. It is a protein with many functions related to the attachment of the virion to the target cell receptor, the enzymatic cleavage of sialic acid, and the promotion of viral fusion. The large protein is the largest in the viral genome, consisting of 2204 amino acids and having a molecular weight of 250 kD. This protein synthesizes viral mRNA and assists in the replication of genomic RNA (Ganar *et al.*, 2014; Phale, 2018; Fellahi and Boudouma, 2021).

Accessory proteins, V and W, are proteins derived from the P gene and translated from alternative mRNAs produced by RNA editing during P gene transcription. The V protein consists of 239 amino acids with a molecular weight of 36 kD. It is an interferon antagonist and plays an important role in NDV virulence. Finally, the role of the W protein in the NDV replication cycle has not yet been established (Ganar *et al.*, 2014; Nurziah *et al.*, 2022).

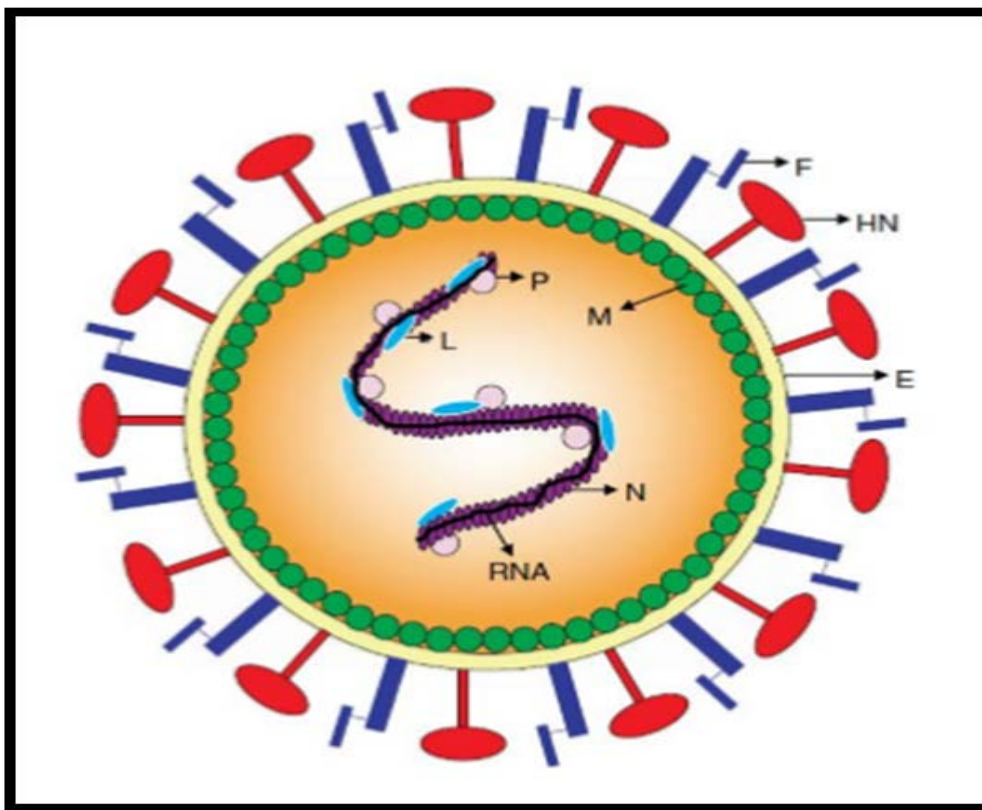


Figure (2-1): Paramyxovirus structural proteins (from left to right): nucleocapsid (N), phosphoprotein (P), large polymerase (L), matrix (M), fusion(F),hemagglutinin-neuraminidase(HN). (Dimitrov *et al.*, 2016).

2.4. Biological properties of paramyxoviruses

2.4.1 Hemagglutination and neuraminidase activity

The hemagglutinin-neuraminidase (H-N) protein binds to receptors on the surface of red blood cells (RBCs), allowing the ND virus and other paramyxoviruses to agglutinate RBCs. This property, together with the unique inhibition of agglutination by antisera, has been shown to be useful in the diagnostic process (UI-Rahman *et al.*, 2022).

Normal chicken red blood cells (RBCs) are used in hemagglutination (HA) tests; however, the ND virus triggers agglutination in cells from all amphibians, reptiles, and birds. A study by Jordan *et al.* (2018) found that although all ND virus strains tested were able to agglutinate RBCs from humans, mice, and guinea pigs, their ability to agglutinate RBCs from cows, goats, sheep, pigs, and horses varied.

The neuraminidase enzyme is included in the hemagglutinin-neuraminidase molecule of all Rubula viruses. Agglutinated RBCs are released from their adhesions at a slow rate by the enzyme. Hemagglutination activity describes the ability of the NDV particles to clump red blood cells. This takes place in RBCs when HN glycoprotein attached to sialic acid receptors on their membrane. In order to inhibit this response, antisera have been used, which has been shown to be helpful in the identification of several diseases (UI-Rahman *et al.*, 2022). The neuraminidase enzyme, which disassembles the host cell membrane and frees the viral particle, is also produced by the HN glycoprotein. This is brought about by a breakdown in the sialic acid receptor (Jordan *et al.*, 2018).

2.4.2 Antigenicity

Antigenicity or the presence of antibodies is defined as few antigenic differences have been found using either viral neutralization or agar gel diffusion tests across different strains and isolates of NDV, suggesting that NDV isolates may be regarded as single antigenically homogeneous group. The use of monoclonal antibodies (MABs) provides a unique method for identifying NDV strains and isolates based on antigenic differences (Aldous and Alexander, 2001).

2.4.3 Virus entry

The envelope proteins of a virus regulate its ability to enter a host cell. The HN protein is responsible for cellular adhesion, while the F protein is essential for cellular fusion. HN proteins need sialic acid residues on the surface of the host cell in order to bind. The neuraminidase activity subsequently exerts helps viruses exit the cell by destroying their sialic acid receptors (Bose *et al.*, 2014). The fusion protein plays a key role in the pathogenesis and virulence of the virus since the virus's ability to enter a cell is reliant on cellular proteases cleaving the inactive F protein. Once the virus envelope has fused with the host cell membrane, the viral nucleocapsid is released into the cytoplasm (Ganar *et al.*, 2014).

2.4.4 Replication

Once the virus enters the cell in a negative sense, its genome is transcribed in the cytoplasm. The viral leader RNA and individual genes are transcribed by the RNA-dependent RNA polymerase into 5'-capped and 3'-polyadenylated messenger RNAs along a transcription gradient. The reason is that more mRNA transcripts are produced from genes nearer to the

promoter region than genes close to the 5' end. The subsequent step is the translation of the different mRNAs into viral proteins (Wignall-Fleming *et al.*, 2019).

Once a sufficient quantity of viral proteins has been synthesized, transcription stops and replication begins. A complete anti-genome of negative sense RNA is produced during replication. Most paramyxoviruses adhere to the rule of six because each N subunit is associated with six bases of genomic RNA. Because of this trait, the sizes of all known NDV genomes are divisible by six (Munir, 2016).

The spread of NDV in the infected organism occurs in several steps. It begins with the penetration of the virus into the host cell via surface glycoproteins (HN and F). The HN protein binds to sialic acid-containing cell receptors on the host cell surface, while the F protein mediates viral entry into the host cell. After the fusion of the viral envelope with the host cell membrane, the viral nucleocapsid is released into the cytoplasm where the transcription and translation phases begin.

During this phase, the negative-sense RNA genome is transcribed into positive-sense mRNA, which is then translated into viral proteins. The mRNA of individual genes is synthesized and used as a template for the synthesis of negative-sense genomic RNA. Transcription of genomic RNA into messenger RNA is carried out by the viral transcriptases (P and L) associated with the nucleocapsid.

After the transcription and translation phases, the newly formed viral particles undergo budding and release. The M protein of NDV is essential for the assembly and budding of viral particles (Mao *et al.*, 2022). Its nuclear

localization could promote viral replication, increase viral RNA synthesis, and inhibit host cell replication (Duan *et al.*, 2019).

The F protein synthesized as a non-functional precursor, F0, is cleaved into F1 and F2 by host cell proteases. Viral proteins synthesized in the host cell are transported and incorporated into the cell membrane. Once the nucleocapsids are incorporated into the modified regions of the cell membrane. New viral particles bud at the cell surface, taking with them part of the cytoplasmic membrane. Finally, the HN protein catalyzes the cleavage of sialic acid in the cell receptor and allows the release of the virus (Ganar *et al.*, 2014; Mao *et al.*, 2022). Infection can also occur through receptor-mediated endocytosis (Sánchez-Felipe *et al.*, 2014). Cellular cholesterol may be required for optimal cell entry into the NDV infection cycle (Martín *et al.*, 2012).

2.4.5. Assembly and release

The N protein forms a helix with the RNA, and the P and L proteins are subsequently integrated into the host cell's cytoplasm. Following their transport to the plasma membrane, the nucleocapsids get attached to the surface glycoproteins F and HN through M protein. The viral envelope is made when the virus emerges from the host cell (El Najjar *et al.*, 2014).

2.5. Infection mechanism

The entire process of NDV infecting host cells from completing its replication to producing infectious progeny viruses, relies on major structural proteins. The infectivity of NDV depends on the cleavage of the F protein precursor F0 to the F1–F2 subunits. Therefore, the amino acid

sequence of the F protein cleavage site is considered to be the main determinant of infection.

HN protein is related to virus attachment and entry into the host cell. The attachment stage of NDV-infected cells is through the combination of HN glycoprotein on the surface of the virus and sialic acid-containing receptors on the host cell surface to infect respiratory epithelial cells, which is the first step of NDV infection (Burman *et al.*, 2020).

Then, the F protein of the viral envelope is responsible for the fusion of the viral envelope and the cellular plasma membrane to allow the entry of the nucleocapsid into the cell cytoplasm. In addition, infections also occur through receptor-mediated endocytosis and sometimes caveolae-dependent endocytosis. Simultaneously, the viral RNA is encapsidated by the NP protein to form an N-RNA template, which is mediated by the P protein and catalyzed by the L protein to form an active RNP complex to enter the cytoplasm.

Following its entry into the host cell cytoplasm, the M protein is separated from the polymerase complex to transcribe the viral genome. The negative-sense RNA genome is transcribed into positive-sense mRNA, which is then translated into viral proteins (Jadhav *et al.*, 2020). The M protein is required for the assembly of virus particles and the budding process of progeny (Takimoto and Portner, 2004; Pantua *et al.*, 2006). Finally, the HN protein promotes the detachment of the virus from the cell and removes sialic acid molecules from progeny virions to prevent self-aggregation during budding (Mao *et al.*, 2022).

2.6. Resistance, virulence, and pathogenicity

The NDV can survive for long periods at (18 to 23.5 °C), especially in feces (Getabalew *et al.*, 2019; Dzogbema *et al.*, 2021). However, it is inactivated at high temperatures (56 °C for 3 hours or 60 °C for half an hour). Also it is inactivated by an acidic pH ≤ 2 . Finally, it is sensitive to detergents, lipid solvents, formaldehydes, and oxidizing agents (Getabalew *et al.*, 2019). The pathogenicity of NDV depends on the virus strain.

the activation of the fusion protein (F) by host cell proteases is the primary determinant of virulence (Ganar *et al.*, 2014). An inactive precursor glycoprotein (F0) is produced during replication. It must be cleaved into F1 and F2 polypeptides to exhibit virulence and become infectious (Worku and Teshome, 2020).

The virulence of the virus is attributed to the cleavability of the F0 molecule, and it is influenced by the nature of proteases present in host tissues and organs. Molecular studies of the F0 glycoprotein site have revealed that the sequence of the F cleavage site between positions 112 and 116 primarily determines the virulence of NDV isolates (Puro and Sen, 2022).

Virulent viruses are cleaved by furin-like proteases found in many tissues and organs, allowing them to cause lethal systemic infections. In contrast, trypsin-like proteases can only cleave low-virulence viruses in limited areas, such as the respiratory and intestinal tracts (Phale, 2018). According to Heiden *et al.* (2014), regions of the F protein modulate virulence other than the polybasic cleavage site.

The development of reverse genetics over the past two decades has allowed researchers to modify NDV genetically and study the individual contribution of genes and genome regions concerning its virulence (Dortmans *et al.*, 2011). According to Samal *et al.* (2011), the conserved glutamine residue at position 114 in the cleavage site of the F protein is important in NDV replication and pathogenicity. In addition, the replacement of isoleucine at position 118 with valine around the fusion cleavage site reduces the pathogenicity of NDV.

Additionally, mutations in the F protein glycosylation site can lead to a hyperfusogenic virus that can increase the virulence of NDV strains (Samal *et al.*, 2012). Mutation of the cytoplasmic domain of the F protein can lead to the creation of a new hyperfusogenic virus that may increase viral replication and pathogenicity (Samal *et al.*, 2013).

Other studies found that the amino acid residue located on the site 430 of the HN protein of NDV can influence viral fusion capacity by promoting F protein cleavage (Chen *et al.*, 2021). In addition, ubiquitination on lysine 247 of the NDV matrix protein enhanced viral replication and virulence by promoting nuclear-cytoplasmic trafficking (Peng *et al.*, 2022).

2.7. Pathology

ND infections can be led to structural alterations in infected tissues and organs called lesions. The lesions are proportional to the tissue, which is damaged by the causative agent. The symptoms of the illness in animals may be logically explained by studying the lesions if the therapy is not administered (Hussein *et al.*, 2019). The birds infected with the ND virus, the severity of the illness as well as the kinds and locations of gross lesions

depend on the strains and pathogenicity of the infecting virus, the host, and other variables (Jatav and Verma, 2021).

Hemorrhagic lesions in the gut of infected birds are to differentiate the viscerotropic velogenic ND virus from the neurotropic velogenic ND virus (Tudeka *et al.*, 2022). The bleeding sores manifest most noticeably in the proventriculus, caeca, and small intestine mucosa. Necrosis of the intestinal wall or lymphoid tissue, such as caecal tonsils and Payer's patches, seems to be the underlying cause of these conditions. Furthermore, it has been stated that ND virus infection in birds does not cause gross CNS lesions (Kabiraj *et al.*, 2020).

Mucosal hemorrhages and significant tracheal congestion are two types of lesions that may affect the respiratory system. Secondary bacterial infections often accompany symptoms like air sacculitis and air sac thickening due to catarrhal or caseous exudates. Velogenic viruses infecting laying chickens and turkeys cause egg yolk peritonitis. Follicles in the ovaries tend to be weak and deteriorate. Reproduction system hemorrhages and discolorations (Suarez *et al.*, 2020).

The specific pathotype of the ND virus causes different clinical and histological findings. Lymphoid and CNS tissues are particularly susceptible to infection by velogenic viruses. Bloody intestinal lesions are a common result of infection with viscerotropic, velogenic pathotypes; these lesions are caused by the necrosis of lymphoid tissue. In particular, the caecal tonsils and the small intestine are frequent sites for fibrinonecrotic lesions (Saravi *et al.*, 2021).

The spleen may be enlarged and mottled, and there may be hemorrhagic foci in the proventriculus, often around lymphoid masses. These physical features are consistent with necrosis of lymphoid tissue with ensuing blood loss and fibrin, as shown on a histology slide. The alterations are linked to viral replication in macrophages, as evidenced by immunohistochemical staining and in situ hybridization (Fawzy *et al.*, 2021).

Eyelid conjunctival lymphocytes and macrophages serve as the first hosts for viral propagation. This is followed by the discovery of positive staining in epithelial cells of the respiratory and gastrointestinal systems, as well as positive staining in mononuclear cells across a variety of organs (Nooruzzaman *et al.*, 2021).

Grossly, neurotropic velogenic strain infections cause few visible lesions, but they may cause serious respiratory complications such as hemorrhagic tracheitis and increased exudate inside the bronchi and trachea. Histological studies demonstrated widespread abnormalities in the brain and spinal cord. Non-suppurative inflammation, primarily perivascular, is present, as well as gliosis and neuronal degeneration.

Lesions are most often observed in the caudal CNS (spinal cord, medulla, and brain stem), making knowledge of lesion distribution crucial for sample collection (Bello *et al.*, 2018). Variances in peripheral replication and the capacity to infiltrate cerebral tissue may be linked to differences in the pathotypes of NDV that may induce lesions in the brain.

Regarding mesogenic viruses, they are similar to velogenic viruses and may infiltrate brain tissue. However, mesogenic viruses replicate at a slower rate. Gross pathological alterations caused by mesogenic viruses in

experimental animals are restricted to splenic enlargement and increased air sac opacity. This is analogous to the development of lymphoid follicles inside the adenoids and spleen. There may be mononuclear infiltration of the myocardium. Furthermore, viruses with a lentogenic origin did not seem to be able to reproduce in neurons (Kabiraj *et al.*, 2020).

Grossly, lentogenic strains caused few symptoms, but histologically, airsacculitis and tracheitis may be present (El-Morshidy *et al.*, 2021). Despite the fact that ND is usually harmless in the wild, it causes neurotropic pathotype lesions in double-crested cormorants in the United States and Canada. Grossly, there are few affected areas, but histological examination revealed gliosis, vacuolation, and mononuclear perivascular cuffing in the brain and spinal cord.

Several histopathology findings in the literature describe alterations that occur during ND infections, and these changes are often associated with virulent pathotypes. Neuronal degeneration, foci of glial cells, perivascular infiltration of lymphocytes, and endothelial cell hyperplasia are all hallmarks of the CNS lesions seen in this case. (León and Chavez, 2019).

Lesions of this kind are uncommon in the cerebrum, but common in the cerebellum, medulla, midbrain, brain stem, and spinal cord. Congestion, edema, and hemorrhaging impact several organs when blood flow is disrupted. The tunica media deteriorates, capillaries and arterioles hyalinize, thrombosis develops in the smallest of blood vessels, and endothelial cells die. Lymphoid tissue disappears as a result of lymphopoietic system regeneration. The liver and necrotic spleen regions are two of the most common sites of hyperplasia produced from mononuclear phagocytic cells (León and Chavez, 2019).

Lymphocytes in the spleen and thymus's cortical region and germinal centers may be destroyed or vacuolated in spots. There is often significant lymphocyte atrophy in the cloacal bursa medulla. Infections with virulent strains of the ND virus leads to hemorrhaging and necrosis of mucosal lymphoid tissue in the intestines (Bello *et al.*, 2018). Furthermore, the epithelia in the respiratory tract may lose their cilia, become congested and oedematous, and invade with mononuclear cells. Broiler tracheitis instances where *E. coli* infection was histologically confirmed and further investigated revealed the presence of lentogenic NDV. NDV lentogenic isolates have been shown to produce conjunctivitis and tracheitis in experimental models. Lymphocyte infiltration was seen histologically, and antigen was detected inside the lesions, both of which are indicative of inflammation (El-Morshidy *et al.*, 2021).

2.8. Genetic diversity and geographic distribution

Several classification schemes to identify and differentiate strains of NDV have been developed. Initially, the schemes were based on the biological properties of the virus (Diel *et al.*, 2012). However, a new classification system has been proposed by Dimitrov *et al.* (2019), which is based on phylogenetic topology, genetic distances, branch support, and epidemiological independence of the virus. This system maintains two classes (I and II) of NDV, identifies three new class II genotypes, and reduces the number of subgenotypes.

The class I NDV isolates are grouped into a single genotype and three subgenotypes, as they exhibit strong genetic relatedness. The isolates have been identified in wild and domestic birds from Africa, Asia, Europe, and America (Rauw *et al.*, 2009). It is worth noting that class I NDV isolates are

generally considered to be of low virulence to chicken, with a few exceptions, such as an isolate, which caused a severe epidemic in Northern Ireland in the early 1990s (Bello *et al.*, 2018), as well as the JS10-A10 and 9a5b strains generated by consecutive experimental passages through chicken (Rehman *et al.*, 2018).

Class II NDV isolates include viruses of varying susceptibilities, including lentogenic, mesogenic, and velogenic strains, resulting in about twenty genotypes due to ongoing virus emergence and evolution (Bello *et al.*, 2018). The most prevalent genotype among all class II viruses in waterfowl is genotype I, which is pathogenic. Additionally, genotype I is further divided into three subgenotypes, namely 1a, 1b, and 1c (Rauw *et al.*, 2009).

Genotype II is neurotropic, but it's included viruses of different pathogenicity (lentogenic, mesogenic, and velogenic). Some viruses belonging to this genotype, including B1, LaSota, VG/GA, and strains not commonly isolated, are used as vaccines (Miller *et al.*, 2010).

Genotype III has been isolated mainly in Southeast Asia, Australia, Japan, the United Kingdom, Zimbabwe, Singapore, and China. All NDV isolates belonging to genotype III are virulent. Their isolates have been found in chickens and domestic waterfowls (Dimitrov *et al.*, 2016). Genotype IV is associated with Newcastle disease epizootics in Europe after World War II (Wehmann *et al.*, 2003).

Genotype V includes four subgenotypes (Va-Vd) and was responsible for an epizootic wave, which started in Western Europe and spread to

Yugoslavia during the 1970s (Wehmann *et al.*, 2003). This genotype has also been isolated in Central and North America and Africa.

Genotype VI has 11 subgenotypes (VIa-VIi) and has been described in pigeons in Asia, Africa, Europe, and South America (Dimitrov *et al.*, 2016; Bello *et al.*, 2018). Genotype VII is commonly found in Asia and Africa (Xue *et al.*, 2017; Naguib *et al.*, 2021) and has been associated with the most recent Newcastle disease epizootics worldwide (Miller *et al.*, 2015).

This genotype has complex genetic diversity since it can be subdivided into nine subgenotypes (VIIa-VIIi; Bello *et al.*, 2018). Genotype VIII has been isolated from South Africa, South Asia, and western China (Cao *et al.*, 2013; Megahed *et al.*, 2020). Virulent and low-virulent isolates of genotype IX have been described in several asymptomatic wild bird species in China (Duan *et al.*, 2014). Virulent strains of genotype X have been identified in Taiwan, Argentina, and the USA. Genotype XI is also virulent, but its distribution is largely restricted to the island of Madagascar (Maminiaina *et al.*, 2010). Genotype XII has been isolated in South America and China (Liu *et al.*, 2013; Chumbe *et al.*, 2017), while the XIII genotype includes three subgenotypes (XIIIa-XIIIc) and has been identified in Asia, Europe, and Africa (Das and Kumar, 2016; Ana *et al.*, 2020).

Genotype XIV includes two subgenotypes. It has highly virulent and has only been found in domestic birds in Africa (Snoeck *et al.*, 2013; Samuel *et al.*, 2013). Genotype XV is isolated in China and included virulent and vaccine strains (Bello *et al.*, 2018). Genotype XVI is strongly related to genotype IV and has been isolated in Europe, Africa, and Asia (Bello *et al.*, 2018). Finally, genotypes XVII and XVIII have two subgenotypes, and they

are highly virulent and are found in West and Central Africa (Snoeck *et al.*, 2013; Bello *et al.*, 2018; Souley *et al.*, 2021).

2.9. Host species

Newcastle disease virus is capable of infecting over 200 different bird species, but the disease infection consequence varies greatly depending on the host species and the virus strain (Rauw *et al.*, 2009). Newcastle disease affects chickens more severely than turkeys, which typically show few clinical signs. Pheasants, partridges, quail, and guinea fowl are also susceptible (Getabalew *et al.*, 2019). Pigeons (Colombiformes) can also be infected with pigeon paramyxovirus 1 (PPMV-1, Ramsubeik *et al.*, 2023). Ducks and geese (Anseriformes) may be infected, but show little or no clinical signs (Rahman *et al.* 2018).

Teal, swans, wild geese, double-crested cormorants, white pelicans, and gulls are susceptible, but they are generally resistant and show little clinical signs of apathy and anorexia (Rasamoelina *et al.*, 2016). Parrots, ravens, sparrows, and kingbirds are susceptible (Rahman *et al.*, 2018). Generally, wild birds and waterfowl are considered reservoir hosts (Getabalew *et al.*, 2019). Newcastle disease can infect a range of non-avian species. Evidence of Newcastle disease infection and detection has been reported in cattle, sheep, mink, hamsters, mice, rabbits, camels, pigs, monkeys, and humans (Ul-Rahman and Shabbir, 2019; Shabbir *et al.*, 2021; Ul-Rahman *et al.*, 2022). Workers in poultry production and processing and vaccine production laboratories are at the greatest risk (Shabbir *et al.*, 2021).

2.10. Mode of dissemination

Transmission of NDV can occur through inhalation or ingestion of virus particles. In the case of a natural infection, the virus multiplies in the respiratory and/or digestive tract of infected poultry, which excretes the virus through airborne or fecal routes (Brown and Bevins, 2017). The released virus particles are inhaled by healthy birds or affect their mucous membranes. Viruses released in feces may contaminate feed and drinking water, causing an infection in other birds when they ingest them (Rauw *et al.*, 2009).

Non-bird species, such as cats, dogs, foxes, and rodents, may play a role in virus transmission because they may spread the virus for up to 72 hours in their feces after ingesting contaminated poultry (Rasamoelina *et al.*, 2016). Similar to insects, rodents, and reptiles may also be potential vectors of NDV, as their susceptibility to infection has been reported (Rasamoelina *et al.*, 2016). Semi-captive or free-living wild birds may act as natural reservoir hosts for NDV and play a considerable role in the spread of the virus (Rahman *et al.*, 2018).

2.11. Clinical signs and lesions

Pathogenicity of the virus strain, host species, host age, secondary infections, stress, environmental conditions, host immune status, viral dose, and route of exposure may play a role in determining incubation time and disease severity (Getabalew *et al.*, 2019). Infection with lentogenic NDV virus strains can range from an unapparent respiratory or gastrointestinal illness to a mild disease in adult chickens.

Infection with mesogenic isolates can lead to the development of nonfatal respiratory disease, decreased egg production, and rare nervous signs, but its mortality is generally low (Abdisa and Tagesu, 2017). Infection with highly virulent velogenic viruses can cause mortality in highly susceptible flocks of up to 100%. Viscerotropic velogenic strains cause acute infection of the gastrointestinal mucosa and result in hemorrhagic lesions and death. Clinical signs resulting from chicken infection included general ill health, respiratory disturbances, greenish or whitish diarrhea, muscle tremors, and paralysis of the extremities. Edema may be seen around the eyes (Caroline, 2022).

Neurotropic velogenic strains of NDV primarily cause respiratory distress, followed by neurological disease and decreased egg production (Abdisa and Tagesu, 2017). Mortality in chickens infected with velogenic NDVs can reach 100% (Al-Azawy and Al-Ajeeli, 2020). NDV lesions are primarily characterized by hemorrhages and ulcerations in the proventriculus and intestine (Rauw *et al.*, 2009).

Furthermore, infection with viscerotropic velogenic NDV can result in severe clinical signs in chickens, including dehydration and emaciation of the chicken carcass, hemorrhagic lesions, and necrotic ulcerations at the tips of the proventricular glands, in the intestine, caecal tonsils, and spleen parenchyma. Additionally, the lungs may show congestive, edematous, and hemorrhagic lesions (Mariappan *et al.*, 2018; Sonkusale *et al.*, 2023).

In the respiratory form of the disease in chickens, lesions are marked initially by congestive or hemorrhagic tracheitis that becomes complicated after a few days of consecutive colibacillosis evolution into fibrinous tracheitis, fibrinous pneumonia, aerosaculitis, pericarditis, and perihepatitis

(Fellahi and Boudouma, 2021). The involvement of the central nervous system in NDV infection can be observed through anatomical manifestations, such as progressive encephalitis, neuronal necrosis, neuronal phagocytosis, the presence of clusters of cells of microglial morphology in the gray matter of the brain, cerebellum, and spinal cord, axonal degeneration, and demyelination lesions (Ecco *et al.*, 2011).

2.12. Diagnosis

Clinical signs cannot provide a reliable basis for diagnosis but can be used as an element of suspicion to guide the diagnosis (Abdisa and Tagesu, 2017). Lesions may also be confused with those of other diseases, including avian influenza (Ganar *et al.*, 2014). However, a definitive diagnosis of Newcastle disease is based on the direct detection of viral antigens, which can be achieved by virus isolation from swabs of live animals or organs taken from cadavers. Virus culture is readily performed on 9-11-day-old embryonated chicken eggs or a wide range of cells, including chicken embryo fibroblast cells, chicken embryo hepatocytes, African vervet monkey kidney cells, and chicken embryo reticulum cells.

After incubation at 37 °C for 4-7 days, the inoculated embryonated chicken eggs are refrigerated at +4 °C. The allantoic-amniotic fluid from the eggs is then tested for NDV using the hemagglutination test. Confirmation of NDV infection can be done by performing a hemagglutination inhibition test or by using molecular methods. Agar gel immunodiffusion technique, fluorescent antibody test, hemolysis test, or identification of viral particle morphology by electron microscopy can also be used to identify NDV, but

none of them provide information on the pathotype of ND (Cattoli *et al.*, 2011).

Serological diagnosis provides indirect evidence of NDV through antibodies that demonstrate infection. The hemagglutination inhibition (HI) test is the most widely used reference and confirmatory test for Newcastle disease serology. The test is based on the agglutination of chicken red blood cells caused by the hemagglutinin of the viral envelope and the inhibition of hemagglutination by specific antibodies. Enzyme-linked immunosorbent assays (ELISAs) are also commonly used for the detection and quantification of antibodies. The antibody detection technique consists of binding antibodies to viral antigens on a microtiter plate.

The viral antigens are captured by other detection antibodies produced in another species against those of the chicken and coupled to an enzyme that catalyzes the reaction to cause a color change. The plate is then read on a spectrophotometer (Dzogbema *et al.*, 2021). The HI test correlated well with the ELISA test (Getabalew *et al.*, 2019). An intracellular cytokine labeling technique was developed to quantify the immune response mediated by T cells (Pinette *et al.*, 2014). There are other serological tests, such as the neutralizing antibody test, the immunofluorescence test, and the colloidal gold immune technique. However, the limitation of serological tests lies in the cross-reactivity between NDV and other homologous avian paramyxoviruses, which can give false positive results (Mao *et al.*, 2022).

Techniques based on molecular biology methods have been developed for the isolation and identification of NDV strains (Dzogbema *et al.*, 2021; Moa *et al.*, 2022). Several laboratory protocols have been developed,

including gel-based reverse transcription polymerase chain reaction (RT-PCR), real-time RT-PCR, restriction enzyme-based methods, and rapid sequencing. In addition, nested PCR, fluorogenic probe-based real-time RT-PCR, ligase chain reaction (LCR), intercalated DNA (SYBR Green), or light-extended fluorogenic primer (LUX) should also be mentioned. All of these assays have shown promising results, but most of them have some limitations, and their ability to detect different virus types needs validation (Cattoli *et al.*, 2011; Mao *et al.*, 2022).

The growth of the gene sequence database over the last decade has revealed the high degree of nucleotide variability in the NDV gene, which is mainly responsible for mismatches between oligonucleotides (primers and/or probes) and cDNA templates. This may well explain the false negative results provided by different real-time RT-PCR protocols (Cattoli *et al.*, 2011). Another real-time reverse transcription isothermal loop amplification (RT-LAMP) method has recently been developed. This assay is faster and more sensitive than real-time RT-PCR, but has specificity only for NDV genotype VII (Selim *et al.*, 2022).

2.13. Pathogenicity testing

Based on calculations of the intracerebral pathogenicity index (ICPI) in one day-old chicks, the intravenous pathogenicity index (IVPI) in six-week-old chickens, or the mean time to death in embryos, the NDV isolates can be characterized as virulent (velogenic), moderately virulent (mesogenic), or less virulent (lentogenic). However, ICPI appeared to be the more accurate and sensitive method compared to the other two methods (Dortmans *et al.*, 2011). The calculation of ICPI is done after intracerebral infection of day-

old chicks with a score ranging from 0 to 2, where 0 is assigned to a normal chick, 1 to a sick chick, and 2 to a dead chick.

This score is assigned daily to each chick for eight days. Finally, the virulence of the virus strain is determined by the average of the scores. Strains are velogenic when the ICPI value is greater than 1.5. They are mesogenic when the ICPI value is between 0.7 and 1.5. Finally, they are lentogenic when the ICPI value is less than 0.7. The IVPI is calculated in the same way as the ICPI, but the six-week-old birds are injected intravenously. The presence of a velogenic strain is indicated by an IVPI greater than 2.5 (Al-Shammari *et al.*, 2020). The mean time to death refers to the average number of hours required for all inoculated embryos to die. If the embryos die within 60 hours, it is an indication of the presence of velogenic strains, while survival of the embryos beyond 90 hours indicates the presence of lentogenic strains (Cattoli *et al.*, 2011).

In vitro, NDV induces plaque formation in embryonic fibroblast culture, the size and morphology which vary according to the virulence of the viral strain. The amino acid sequence of the F protein cleavage site can also be used in differentiating the virulence of NDV isolates (Awad *et al.*, 2020).

2.14. Control

Control of ND must include strict biosecurity, which will prevent velogenic virus strains from coming into contact with poultry and adequate administration of effective vaccines (Dimitrov *et al.*, 2017). In addition, serological monitoring of the immunological status of vaccinated flocks should be performed to assess the antibody response to the administered vaccines (Ahmed and Odisho, 2018). Traditional vaccines (live and

inactivated vaccines) have been widely used for several decades (Dimitrov *et al.*, 2017). However, another generation of vaccines such as recombinant vaccines and antigen-matching vaccines have recently been adopted in some countries. Other vaccine approaches are in the experimental phase (Dimitrov *et al.*, 2017; Nurziah *et al.*, 2022).

The vaccine strains obtained from naturally apathogenic, low-pathogenic, or medium-pathogenic strains (Dimitrov *et al.*, 2017; Hu *et al.*, 2022). These vaccine strains belonged to genotype I (Ulster and V4), genotype II (Lasota and B1), genotype III (Mukteshwar), genotype IV (Herts/33, UK), genotype V (Anhinga, USA), genotype VIII (AF22440), and genotype XV (vaccine strains, China, Bello *et al.*, 2018). With the development of genetic engineering, NDV vaccine strains have emerged as promising vectors for developing effective multipurpose vaccines against pathogens that infect both animals and humans (Kim and Samal, 2016; Choi, 2017). For example, the use of an NDV virus-like particle-based vaccine expressing NDV F protein and influenza virus matrix 1 (M1) protein could protect chickens from a lethal challenge while significantly reducing viral shedding and can also be used as a DIVA strategy to differentiate infected animals from vaccinated animals (Park *et al.*, 2014).

An experiment conducted by Izquierdo-Lara *et al.* (2019) indicated that vaccine strains paired with wild-type XII strains conferred better protection to vaccinated animals and significantly reduced viral shedding. Similarly, Miller *et al.* (2013) in an experimental setting showed that by increasing the homology of the vaccine to a wild-type virus, humoral antibody efficacy levels could be increased.

As the level of humoral antibodies increases in vaccinated birds, the number of infected birds and the amount of virus shed decrease. Results of an experiment conducted by Pandarangga *et al.* (2022) also revealed that viral shedding in chickens is reduced when vaccinated with strains homologous to the infectious challenge strain compared to the vaccine using the heterologous strain (LaSota). Furthermore, in an intranasal vaccination trial in chickens, Zhao *et al.* (2016) found that a DNA vaccine containing the NDV *F* gene encapsulated in hollow nanoparticles induced higher serum antibody (IgA) titers. Results of a comparative study by Zhao *et al.* (2012) also showed that a live vaccine (LaSota strain) encapsulated in chitosan nanoparticles (NDV-CS-NP) induced better protection for immunized specific pathogen-free chickens compared to the live LaSota strain vaccine and the inactivated NDV vaccine.

An encapsulated, inactivated NDV vaccine in chitosan nanoparticles has also been successfully developed (Mohammadi *et al.*, 2021). These studies lay the foundation for the future development of mucosal vaccines encapsulated in nanoparticles. Plant-derived vaccines are in development and offer potential advantages over traditional vaccines, including a significant reduction in viral shedding and the ability to differentiate infected birds from vaccinated birds (Nurzijah *et al.*, 2022; Smith *et al.*, 2023).

2.14.1. Vaccine and vaccination

The most reliable method for preventing ND is still vaccination in conjunction with good hygiene practices. Only a widespread vaccination program can stop an outbreak. Vaccination campaigns are an effective way to quickly and drastically reduce losses due to illness; after an ND vaccination program, mortality in village flocks reduced from 50% to 8%,

and the number of chickens in the area climbed from 900 to 3,500, a rise of 250%. (Muduli *et al.*, 2019).

It has been claimed that vaccination is the sole protection against Newcastle disease in areas where it is prevalent. However, current vaccines on the markets are mostly useful for commercial poultry breeders whose birds are housed in large, single-age, restricted flocks (Habibi *et al.*, 2020).

Heat-sensitive ND vaccines are manufactured in 1,000 or 2,500 dosage multi-dose vials and must be refrigerated (within 'cold chain') from the time they are made until they are given to the hens. In contrast, chickens in villages are grown in tiny flocks of varying ages, and they are allowed to roam freely in their environment. Maintaining the cold chain and paying in foreign currency for commercial vaccinations are also challenges in rural areas (Absalón *et al.*, 2019).

As such, vaccination is a crucial tool in the fight against and prevention of Newcastle disease. Vaccines against ND are now available in the form of both inactivated and live viruses. Additionally, vaccines that can withstand high temperatures are available for use in backyards and backyard chickens in rural areas. From incubation until maturity, it offers flexibility in live vaccine delivery schedules and routes (Campbell *et al.*, 2018).

Vaccines made from oil emulsions of killed viruses are given to the parents before egg production begins. Vaccines against lentogenic viruses are administered in the form of water, eye drops, aerosol, or intranasal spray. In nations where village chickens make up a significant component of the poultry output, a vaccination based on a heat-tolerant V4 strain has been developed for feeding to these birds. Although immunization protects birds

from clinical sickness, it does not stop viral multiplication and shedding, which creates a reservoir of infection (Absalón *et al.*, 2019).

2.14.2. Types of vaccine

2.14.2.1. Live vaccine

Live vaccines may be utilized for mass immunization since they are inexpensive, available in freeze-dried form, simple to give, and safe. Eye drops, beak intranasal installation, or dipping are the ideal methods of administration for lentogenic vaccinations, but intramuscular injection is required for mesogenic vaccines. The use of drinking water and aerosol sprays are also viable options (Ferreira *et al.*, 2020).

Vaccines like Hitchner B1 and La Sota, both of which are made from the lentogenic strain of NDV, are widely used across the world. Some mesogenic vaccinations have been shown to induce illness, most notably in young birds and in cases of concurrent infection with pathogens that exacerbate the sickness. Due to their heat susceptibility, live vaccinations are at a disadvantage under the village management system, where transportation and cold storage facilities are generally insufficient (Martinez *et al.*, 2018).

2.14.2.2. Inactivated vaccine

Inactivated vaccines may be administered in settings where live vaccines would be dangerous and yet nevertheless produce long-lasting protective antibody levels. These vaccines are created by combining adjuvant with B-propiolactone or formalin-treated virulent NDV infective allantoic fluid. Subcutaneous or intramuscular injections may be used to deliver the vaccination (Mayers *et al.*, 2017).

2.14.2.3. Recombinant vaccine

Recombinant vaccines rely on the capacity of one or multiple defined antigens to induce immunity against the pathogen, when administered in the presence of adjuvants or when expressed by plasmids or harmless bacterial/viral vectors. Recombinant protein vaccines permit the avoidance of several potential concerns raised by vaccines based on purified macromolecules, such as the risk of co-purification of undesired contaminants or reversal of the toxoids to their toxigenic forms, if considering diphtheria or tetanus toxoid vaccines, for example. Another fundamental issue overcome by this technology is the complexity involved in obtaining sufficient quantities of purified antigenic components.

However, one of the main challenges in the development of these new strategies of immunization consists of designing vaccines that elicit the appropriate kind of immune response to confer immunity mainly to intracellular pathogens and especially to those that establish chronic, often lifelong infections. For this, the knowledge of the biology of highly conserved antigens involved in pathogenesis and of the immune mechanisms that should be elicited for protection must be obtained to rationally design vaccine strategies that can overcome the low protective immunity naturally generated by infection (Lemaire *et al.*, 2012).

2.14.2.4. DNA vaccine

The direct injection of a naked DNA plasmid into muscle as a vaccine system with the ability to induce an immune response and protection after challenge is now well established, since this approach has been used to express numerous antigens from different pathogens with promising results.

A DNA vaccine (or genetic vaccine as it is also called) consists of a plasmid containing: 1) one origin of replication of *Escherichia coli*, for the amplification of the plasmid; 2) a strong promoter, generally from cytomegalovirus; 3) multiple cloning sites, in which one can insert the gene to be expressed, and 4) an antibiotic as selection marker (Oliveira *et al.*, 1999).

The idea behind the DNA vaccine system is that the antigen can be expressed directly by the cells of the host in a way similar to that occurring during viral infection. As a result, the antigens can be processed as proteins synthesized in the cytoplasm, and the fragmented peptides presented to the immune system by class I MHC molecules. In addition, if the protein is exported or secreted, it can be processed by class II MHC molecules and, as a result, mount a specific antibody response (Ulmer *et al.*, 1993).

2.14.3. Vaccination program

The length of immunity might be affected by the vaccination schedule. Maternal immunity in young chickens is a crucial factor that may vary greatly from batch to batch, farm to farm, and even between individual chickens, and so has a significant impact on vaccination programs. Because of this, one of many tactics is used. Vaccinating birds at one day of age, either by conjunctival instillation or the administration of a coarse spray, is ineffective since most birds are immune to the disease until they are 2–4 weeks old. This will cause some birds to have an active infection that will remain until their mother's immunity has worn out. After 2–4 weeks, a booster immunization is administered (Nagy *et al.*, 2020).

2.15. Inflammatory response induced by Newcastle disease virus

Cytokines are proteins or peptides that activate and regulate other cells and tissues to aid in immunological and inflammatory responses. The structure of cytokines and their function in health and sickness have been described in a plethora of papers, making clear their involvement in mammals (Germolec *et al.*, 2018).

Furthermore, a variety of cytokines have been discovered in chickens and other birds. Despite the limited availability of recombinant cytokines and monoclonal antibodies against avian cytokines, recent advances in technology, such as real-time quantitative PCR, have made it possible to measure the expression of messenger RNA-encoding cytokines without resorting to protein or antibody. This has allowed researchers to explore new avenues in their quest to establish the role of cytokines in illness, therefore enhancing our knowledge of the processes underlying pathogenesis and immunity (Webers *et al.*, 2020).

The use of cytokines to prevent and treat viral diseases in poultry also has great promise. They are investigated as potential new therapeutic agents for treating illnesses. Furthermore, cytokines may be used as vaccine adjuvants to stimulate the immune system in a way that provides protection (Rowley *et al.*, 2019).

One of cytokines's roles is to stimulate and control immune system cell activity. Many different kinds of cells create cytokines; however, these cells differ greatly in the sorts of cytokines they make. Examples of cytokines that may be produced by different cell types include those involved in inflammation generation (so-called proinflammatory cytokines) by epithelial

cells and those involved in the activation and regulation of T helper lymphocytes (Th) in the development of an adaptive immune response by macrophages (Rayes *et al.*, 2020).

All cytokines influence their target cells by binding to specific receptors on their surfaces. This may either stimulate or inhibit the activity of the cell. Based on their function and the cells they are generated by, cytokines have been categorized into a number of distinct classes. Chemokines, interleukins, tumor necrosis factors, transforming growth factors, migratory inhibitory factors, and the smaller chemokines all fall under these categories. On the other hand, there is a lot of crossover across the various classifications (Mitra and Leonard, 2018).

Many cytokines have pleiotropic effects, but their names represent just one of those effects (like TNF's moniker, which comes from its capacity to act on tumors). It may be more useful to understand the nature of cytokines' general activity if we classify them generically according to their activity (Konjević *et al.*, 2019).

Interleukin 6 (IL-6), Interleukin-1 beta (IL-1 β), and Interferon-gamma (IFN- γ) are multifunctional cytokines generated by a variety of cell types and are involved in acute-phase responses, immunological control, and haematopoiesis. Serum amyloid A, C-reactive protein (CRP), and matrix metalloproteinase-9 (MMP-9) are all examples of acute phase proteins which are produced as part of an inflammatory response to infection or other stressors in animals (Aliyu *et al.*, 2022). C-reactive protein is an acute inflammatory protein that increases at sites of infection or inflammation. CRP is a protein made by the liver and sent into the bloodstream.

Matrix metalloproteinase-9 is part of a larger family of proteins known as matrix metalloproteinases. This enzyme is produced by various types of cells; cytokine-activated leucocytes such as monocytes and neutrophils are major producers of this enzyme. These proteins help to break down and remodel the tissues in the body. This is a normal and necessary process that happens all the time, whether it's to heal a wound, grow new tissue, or even during the normal turnover of cells. MMP-9, in particular, is like a tiny, precise pair of scissors that can cut through certain materials in the body's tissues, allowing them to change and adapt as needed (Nandi *et al.*, 2020).

Previous studies have revealed that Interferon-gamma (IFN- γ) can halt viral replication, enhance activation of both MHC-I and MHC-II molecules, and assist in antigen presentation and processing as well as the removal of intracellular pathogens (Yeh *et al.*, 1999; Schultz and Chisari, 1999; Kaiser, 2010). Moreover, IFN- γ activates T-helper I-type immune responses (Fensterl and Sen, 2009). IFN- γ and its adjuvant characteristics have potential antiviral activity against avian viral pathogens such as avian influenza virus (AIV), NDV, and Marek's disease virus (MDV) (Sawant *et al.*, 2011; Susta *et al.*, 2013; Haq *et al.*, 2015).

Interleukin-1 beta (IL-1 β) is an essential component of the inflammatory process during viral infections. Since IL-1 β is a central mediator of the inflammatory reaction during infections, its increases could lead to the synthesis and release of IL-6, intercellular adhesion molecules, and vascular cell adhesion molecules, which activate lymphocytes and promote the infiltration of leukocytes and eosinophils into the site of inflammation. An appropriate amount of IL-1 β expression can repair damage and reduce viral proliferation. However, excessive IL-1 β expression exacerbates the degree

of inflammation, thereby increasing morbidity and mortality (Peiro *et al.*, 2017). Finally, Interleukin-6 is released by monocytes and macrophages in response to other inflammatory cytokines that include IL-11 and tumor necrosis factor (TNF) beta. Besides being an immune protein, IL-6 is also a pyrogen and is responsible for fever in autoimmune, infectious, or non-infectious diseases. IL-6 is produced in the body wherever there is inflammation, either acute or chronic, including situations such as trauma, burns, cancers, and infection (Fain 2010).

Chapter Three

Materials and Methods

3. Materials and Methods

3.1. Materials

3.1.1. Instruments and equipment

The instruments and equipment used in this study are listed below (Table 3-1).

Table (3-1): Instruments and equipment have been used in this study

No.	Instruments and equipment	Manufacture	Origin
1.	Gel electrophoresis	Shandod Scientific	UK
2.	High speed cold centrifuge	Eppendorf	Germany
3.	Kern PFB balance	Kern & Sohn	Germany
4.	Micropipettes 5-50, 0.5-10,100-1000µl	VWR	USA
5.	7300 Real-Time PCR	Applied Biosystems	USA
6.	Nanodrop lite spectrophotometer	Bio-Rad	USA
7.	Optimus 96G thermal cycler	QLS	UK
8.	Pipettes	DARWELL	China
9.	UV Transilluminator	ATTA	Korea
10.	Vortex	Mammer/CYAN	Belgium

3.1.2. Chemicals, biological materials, and kits

The chemical reagents and kits as well as their manufacturer used in the study have been indicated in the table below.

Table (3-2): Chemicals and reagents have been used in the study

No	Name	Company	Country
1.	10X TAE (Tris Acetate EDTA) buffer	Promega	USA
2.	2-Mercaptoethanol	Sigma	Germany
3.	Absolute Ethanol	Scharlau	Spain
4.	Agarose LE	Intron	Korea
5.	Chlorophorm	Sigma-Aldrich	Germany
6.	Ethidium Bromide	BioBasic	Canada
7.	Free nuclease water	Bioneer	Korea
8.	GoTaq® G2 Green Master Mix	Promega	USA
9.	Isopropanol	Sigma-Aldrich	Germany
10.	Ladder 100bp and Ladder 50bp	Intron	Korea
11.	NDV Ag test kit	Bionote	Korea
12.	Power SYBR™ Green PCR Master Mix	Applied Biosystems™	/USA
13.	RNeasy miniextarction Kit (50)	Qiagen	USA
14.	Safe-Green 100bp Opti-DNA Marker	Abm	Canada
15.	SuperScript™ III Reverse Transcriptase	Invitrogen	USA
16.	Taq PCR Master Mix Kit	Qiagen	USA
17.	TBE buffer 10X	BioBasic	Canada
18.	TRIzol	Thermo-fisher Scientific	US
19.	Viral Gene-spin™ Viral DNA/RNA Extraction Kit	Intron	Korea

3.2. Study design and collection of the samples

Clinical and pathological indications associated with Newcastle disease were observed in about 15 flocks across Thi-Qar Province (Nasiriyah, Shatrah and Refae). The flocks were vaccinated against NDV with killed and attenuated LaSota strain. The majority of flocks showed clinical signs, such as loss of appetite, coughing, gasping, nasal discharge, watery eyes, bright green diarrhea, and convulsions. 8-12 suspected samples were collected from each flock. The number of samples were 83 samples (suspected). 40 samples as (control) birds collected from non-infected area at the age from 20 and 35 days. At the beginning, a rapid NDV test was utilized to confirm the infection. The samples were collected during the period from October 2023 to February 2024. Blood, trachea, lung, and cecum were collected. Two hundred microliters of blood were collected in a 1.5 ml tube containing 1 ml of Trizol for RNA extraction. Also, serum was collected from blood samples and frozen at (-20 °C). Trachea, lung, and cecum were snap frozen in liquid nitrogen (-196 °C). The design of the study is shown in the figure (3-1).

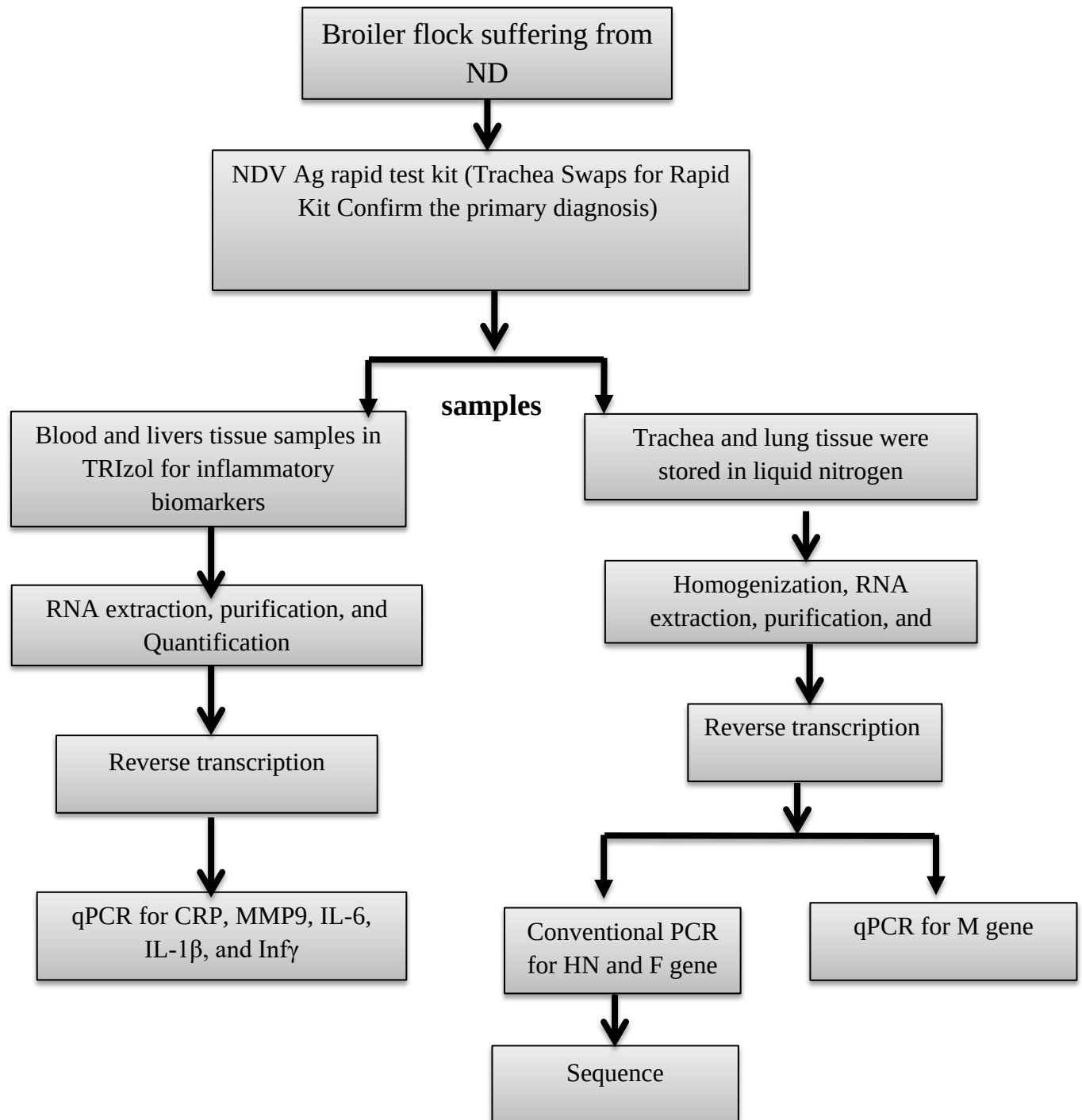


Figure (3-1): The study design

3.3. Diagnosis

3.3.1 Primary (suspected) diagnosis

The primary diagnosis was performed depending on the several criteria, such as the case history, including whether the ND infection occurred on the farm previously, clinical signs, and postmortem lesions.

3.3.2 Immunological diagnosis

3.3.2.1 NDV rapid kit (Bionote, Korea)

The NDV rapid kit was used to diagnose the NDV infection in the flocks. The kit was purchased from Bionote, a Korean company. The NDV Rapid Kit is a qualitative detection of NDV antigen in serum. The kit shows two lines, which are the test (T) and the control (C) lines, on the surface of the device. The control line is a reference line that ensures the test is performing properly. The control line has to appear at all times after the test has been performed. If the NDV antigens exist in the sample, a purple test line would appear in the result window. The NDV antigen in the sample may be recognized by these antibodies with high reliability.

3.3.3. Molecular diagnosis

3.3.3.1. Reverse transcription–quantitative polymerase chain reaction for inflammatory markers using.

3.3.3.1.1. RNA extraction (Qiagen RNA easy mini kit, USA)

A. Homogenization and phase separation

1. 200 μ L of blood or a small piece of liver (about 30 gram) was homogenized in 1 ml of Trizol.
2. Incubate the homogenized sample for 5 minutes at room temperature.
3. Add 0.2 mL of chloroform per 1 mL of Trizol reagent.
4. Cap the sample tubes securely and shake vigorously by hand for 15 seconds. Incubate the samples at room temperature for 3 minutes.
5. Following the incubation, centrifuge the samples at 12000 g for 15 minutes at 4 °C.
6. Following centrifugation, the mixture separates into a lower red phenol-chloroform phase, an interphase, and a colorless, upper aqueous phase. RNA remains exclusively in the aqueous phase (which is about 60% of the volume of Trizol reagent used for initial homogenization).
7. Transfer the aqueous phase containing RNA into a new tube.

B. RNA precipitation

1. 0.5 mL of isopropanol per 1 mL of Trizol reagent added to the tube containing the aqueous phase and mix by pipetting up and down gently. (Do not vortex).
2. Incubate the samples for 20 minutes at -20 °C, then centrifuge at 11,600 x g for 10 minutes at 4 °C.
3. The RNA precipitate forms a translucent gel-like pellet on the side and bottom of the tube.

C. RNA wash

1. Decant the supernatant to a clean, labeled 1.5-mL microcentrifuge tube and set aside.
2. Wash the RNA pellet once with 75% ice-cold ethanol. Use 1 mL of 75% cold ethanol per 1 mL of TRIzol reagent used.
3. Mix by inversion. Centrifuge at 10,000 g for 5 minutes at 4 °C.

D. Resuspending the RNA

1. The supernatant was removed with a pipette. Quickly spin in a capsule centrifuge to collect any remaining 75% ethanol at the bottom. Remove as much of the remaining ethanol with a pipette and air-dry the RNA pellet by leaving the tubes open on the counter for approximately 15-30 minutes. When the pellet is dry, there must be no visible ethanol in the tube. Do not over-dry the pellet, as it may be difficult to re-dissolve.
2. Re-suspend the RNA pellet in 86 µL of nuclease-free water by pipetting up and down gently and incubating in a 60 °C water bath for 10 minutes (you can incubate longer if necessary, up to one hour). Flick (do not vortex) gently to mix and quickly spin to collect the solution.

E. DNase treatment of RNA

1. Added 0.1 volume (5.6 µL) of 10X DNase I buffer and 1 µL of DNase I (2 units) to the RNA. Mixed gently by flicking the tube (DO NOT VORTEX) and incubate in a 37 °C water bath for 20 minutes.
2. Added 0.1 volume of DNase Inactivation Reagent (6.17 µL) to the RNA. (Make sure to vortex the DNase Inactivation Reagent before addition to the RNA.) Flick (do not vortex) the RNA tube to disperse

the reagent. Incubate for an hour at 37 °C. Flick the tube once more during the incubation to redisperse the DNase Inactivation Reagent.

3. Centrifuged at 14000 rpm for 1 minute at 4°C. Transfer the supernatant RNA solution to a 1.5-mL microtube.

F. RNA purification

1. Prepared a mixture of RLT with 2 mercaptoethanol (1 ml RLT/10 µL 2 mercaptoethanol). Add 350 µL of the mixture to the RNA tube and mix by pipetting.
2. Added 250 µL of ethanol to the tube and mix by pipetting.
3. Transfer the mixture to the mini spin column placed in a 2 ml tube and centrifuge at maximum speed for a minute.
4. Discard the flow through and transfer the mini spin column to the new 2 ml collecting tubes provided with the kit.
5. Added 500 µL of RPE to the mini spin column and centrifuge at maximum speed for one minute. Discard the flow.
6. Added 500 µL of RPE to the mini spin column and centrifuge on maximum speed for one minute. Discard the flow.
7. Centrifuged the mini spin column at maximum speed for two minutes.
8. Place the mini spin column in a new 1.5-ml collection tube, add 40 µL of RNA-free water, and centrifuge for 1 minute.
9. Added 30 µL of RNA-free water and centrifuge.
10. Immediately Qubit the extracted RNA or store it at -20 or -80 °C until further analyses.

G. Determination of RNA concentration

- 1- Diluted 2µl of RNA in 8µl of RNA-free water and mix well in an Eppendorf tube.
- 2- Determined the RNA concentration using a nanodrop.
- 3- If the 260/280 ratio between 1.8 and 2.2, the RNA is pure and acceptable for further analysis.

3.3.3.1.2. Reverse transcription (cDNA synthesis)

The following procedure is performed in 40 µl using SuperScriptIII cDNA synthesis Kit (Invitrogen/USA)

- 1- RNA (2 µg) was incubated with 2 µl of oligo(dT) and 2 µl 10 mM dNTP in the Eppendorf tube at 65 °C for 5 minutes.
2. Then, the mixture was transferred to the ice for 2-3 minutes.
3. After that, 20 µl of cDNA synthesis mix (10X RT Buffer, 25 mM MgCl₂, 0.1 M DTT, RNaseOUT, and Superscript III RT) was added and incubated at 50 °C for 5min.
4. Inactivated the reaction by heating at 85°C for 5 minutes.
5. The RNA was removed by adding 2 µl RNase H and incubated at 37 °C for 20 min.
6. Store the cDNA at -20 °C or -80 °C for later use.

3.3.3.1.3. Quantitative polymerase chain reaction (qPCR)

1. The reaction was performed in 30 µl in 96-well PCR plates.
2. Power Syber® Green Mastermix 2x (ready to use) was used.
3. Primers (forward and reverse) were added to the PCR in the table.
4. cDNA, about 25 ng per well, was loaded into each well.

5. The PCR conditions applied in the current study are shown in the table below.

Table (3-3): Components for real time PCR reaction (Kadhim *et al.* 2021)

Components	Concentration	Volume (30 µl)
Power Syper® Green Master Mix	2X	15 µl
Forward primer	10 µM/µl	2 µl
Reverse primer	10 µM/µl	2 µl
ddH ₂ O	-	8 µl
cDNA	40ng	3µl

Table (3-4): Real time PCR conditions (Kadhim *et al.* 2021)

Phase	T _m (°C)	Time	Cycles
Initial denaturation	94°C	5 min	1X
Denaturation	94°C	30 sec.	40X
Annealing	50-58°C	30 sec.	
Extension	72°C	1 min	
Final extension	72°C	5 min	1X

3.3.3.2. Conventional polymerase chain reaction for HN and F gene

3.3.3.2.1. Viral RNA extraction from VTM (Using Viral Gene-spin™ Viral DNA/RNA Extraction Kit) was done according to company instructions as following steps:

1. 500 µl of Lysis buffer was added to 300 µl of serum in 1.5 ml tube, vortexed for 15 seconds, and incubated at 15-25 °C for 10 minutes.
2. Added 20 µl of Proteinase K Solution (20 mg/ml) and incubate at 55 °C for 10 minutes.
3. Then, 700 µl of binding buffer was added to the mixture and vortexed to ensure mixing.
4. Load the mixture to the spin column and insert into the 2-ml collecting tube, centrifuge at 13,000 rpm for 1 min, and discard the flow-through.
5. Washing buffer-A (500 µl) was added to the column and centrifuged at 13,000 rpm/ 1 minute. Then, the flow through was discarded.
6. Step 5 was repeated using washing buffer-B.
7. After that, the spin column was centrifuged for 2 minutes at 13000 rpm to get rid any residual solution left in the column.
8. The RNA in the spin column was eluted using 40 µl of Elution buffer in 1.5ml RNase free tube. Then the elution was repeated using 30 µl to maximize the RNA yield.
9. RNA concentration was measured using the nanodrop.

3.3.3.2.2. cDNA synthesis (using Superscript® III reverse transcriptase from Invitrogen)

1. First-strand cDNA was synthesized in 40 µl from total RNA for each sample treated with DNase I (Ambion, Austin, TX, USA) using Superscript® III reverse transcriptase (Invitrogen) according to the manufacturer's protocol.
2. RNA was incubated with 2µl of Oligo (DT) and 2µl of dNTPs at 65 °C/ 5 min.
3. Then, the mixture was transferred to the ice for 2-3 min.
4. After that, 20 µl cDNA synthesis mixed (10X RT Buffer, 25 mM MgCl₂, 0.1 M DTT, RNaseOUT, and superscript III RT) was added and incubated at 50 °C/5min.
5. Then, the reaction was terminated by 85 °C/ 5min, and the RNA was removed by adding 2 µl RNase H and incubated at 37 °C/20min.

3.3.3.2.3 PCR condition (Using *Taq* PCR Master Mix Kit (2x) from Qiagen)

1. The reaction was performed in 30 µl in 96-well PCR plates.
2. ***Taq* PCR Master Mix** (ready to use) was used.
3. Primers (forward and reverse) were added to the PCR in the table.
4. cDNA, about 50 ng per well, was loaded to each well.
5. The PCR conditions applied in the current study are shown in the table below.

Table (3-5): Components for conventional PCR reaction(Kadhim *et al.* 2021)

Components	Concentration	Volume (50 μ l)
Taq PCR Master Mix	2X	25 μ l
Forward primer	10 μ M/ μ l	5 μ l
Reverse primer	10 μ M/ μ l	5 μ l
ddH ₂ O	-	10 μ l
cDNA	50ng	5 μ l

Table (3-6): Conventional PCR conditions (Kadhim *et al.* 2021)

Phase	T _m (°C)	Time	Cycles
Initial denaturation	94°C	3 min	1X
Denaturation	94°C	60 sec.	30X
Annealing	50-60°C	60 sec.	
Extension	72°C	1 min	
Final extension	72°C	10 min	1X

3.3.3.2.4. Agarose gel electrophoresis of DNA

The electrophoresis was performed for conventional PCR products in order to visualize the PCR output and observe the size band, as well as compare the band with a ladder that is well known for the size of the base pair. The DNA concentration was used to be measured on a 1-2% agarose gel. The gel was prepared according to Sambrook *et al.* (1989).

In brief, agarose (1 g) was dissolved in 100 ml of 1X TAE by heat using a microwave until it completely dissolved. After that, the mixture was allowed to cool (45°C–50°C), and 10 µl of ethidium bromide was added. The gel was poured into the gel camper containing a comb and set aside for about 30-45 minutes to let the gel completely become solid.

Then, the comb was removed, and the gel was loaded into the electrophoresis chamber. Thereafter, the electrophoresis tank was filled with 1X TAE, and the gel was covered by the electrophoresis buffer (TAE), followed by loading the samples and ladder. The ladder (5µl of Safe-Green 100bp Opti-DNA Marker) was placed on the first well. Then, the power supply (90 volts, constant current, 45 minutes) was applied. At the end of electrophorsis, the gel was transferred to the dark room to visualize the band on a 320nm UV light source.

3.3.3.3. Primers

Primers used in the current study were provided kindly by Dr. Sun at the Health Lab/University of Arkansas (Sun *et al.*, 2023). Also, the primers were checked using primer design tools (NCBI). The primers were received as lyophilized. Upon receiving, ddH₂O was used to dissolve them to get the final concentration of 100 µM/µl, which served as a stock solution that was

stored at -20 C. While the working concentration was 10 $\mu\text{M}/\mu\text{l}$, Also, the primer concentration was measured using nanodrops. The primers used in this study are listed in the Table 3–7.

Table (3-7): Primer sets used in the study

Target gene	Sequence (5'-3')	Product size	Reference	Accession number
F gene-NDV	F: ATGGGCGYCCAGAYCTTCTAC R: CTGCCACTGCTAGTTGTGATAATCC	565	(Wang <i>et al.</i> , 2006)	KF494201.1
M gene-NDV	F: ATCTATCTGTTCTGGGCTCAGTC R: GGCTGTCCCACTGCTAGAGA	107	(Zeng <i>et al.</i> , 2021)	MZ306221
HN gene-NDV	F: GAGCTGCAAACAACAGTGGG R: TCTCCGTGACTTTCGAGCAC	416	(Sun <i>et al.</i> , 2023)	OQ641473.1
CRP	F: ATACGTCGCCTTCCACATCC R: CGTTGCCCACCACGTA	149	(Sun <i>et al.</i> , 2023)	NM_001313720.3
Mmp9	F: CCAAGATGTGCTCACCAAGA R: CCAATGCCCAACTTCTCAAT	108	(Tang <i>et al.</i> , 2019)	NM_204667.2
IL6	F: TGGTGATAAATCCCGATGAAG R: GGCCTGAACTCCTGGTCT	191	(Sun <i>et al.</i> , 2023)	NM_204628.2
Il1 β	F: GCATCAAGGGCTACAAGCTC R: CAGGCGGTAGAAGATGAAGC	131	(Sun <i>et al.</i> , 2023)	XM_046931582.1
Infy	F: AGCTGACGGTGGACCTATTATTGT R: CGGCTTTGCGCTGGATTC	260	(Tang <i>et al.</i> , 2019)	NM_205149.2
Gapdh	F: GACGTGCAGCAGGAACACTA R: CTTGGACTTTGCCAGAGAGG	128	(Kadhim <i>et al.</i> , 2021)	NM_204305

3.4 DNA sequencing method

Fusion (F) and haemagglutination neuraminidase (HN) gene confirmation sequencing and phylogenetic tree analyses were carried out by using DNA sequencing. The positive samples were submitted to a MacroGen company in Korea to have the PCR output sequenced using AB DNA sequencing technology. Clustal W alignment analysis and the Molecular Evolutionary Genetics Analysis, version 11.0 (Mega 11.0) were used in the DNA sequencing analysis for multiple sequence alignment studies of the partial fusion (F) protein gene and hemagglutinin neuraminidase (HN).

3.5 Statistical analysis

Statistical analyses of gene expression data were performed using JMP® Pro 18.0 (SAS Institute Inc., NC). A normal distribution was first tested, and thereafter, a student t-test was used to evaluate significant infection effects on the inflammatory biomarkers. A mean separation test Tukey's Kramer HSD procedure, was used to find significant differences between the infection group and control group. Fold changes of gene expression for each gene in the infected group compared with controls. The data are presented as the mean $\pm$ SEM. A probability level of $p \leq 0.05$ was considered statistically significant.

Chapter Four

Results

4. Results

4.1. Sampling and initial diagnosis

Despite the fact that all flocks were vaccinated (Newcastle LaSota vaccine) by reliable administrative methods such as subcutaneous injection, spraying, eye drop, or drinking water, the flocks at 3-5 weeks showed a variety of clinical and pathological signs similar to those observed in the ND. The main clinical signs were loss of appetite, coughing, watery eyes, nasal discharge, green diarrhea, and nervous signs. Table 4-1 shows the number of cases with a possible ND virus infection based on digestive, respiratory, and nervous signs.

Table (4-1): Possible infection with ND based on clinical signs

Clinical signs	Possible infection with ND according to symptoms
Digestive signs	17(20.48%)
Nervous signs	12(14.46%)
Respiratory signs	24 (28.92%)
Mixed signs	30 (36.14%)
Total	83 (100%)

4.2 Clinical findings

4.2.1 Clinical signs

Clinical signs were dependent on factors, such as the virus strain, host species, age of the host, co-infection with other microorganisms, environmental stress, and immune status. In chickens, the general symptoms were loss of appetite, listlessness, abnormal thirst, weakness, tracheitis, and conjunctivitis. Respiratory signs can include sneezing, gasping for air, nasal discharge, and coughing, whereas a clear intestinal symptom is greenish, watery diarrhea. Nervous symptoms may consist of paralysis of wings and/or legs, twisting of the head and neck, or complete paralysis (Fig. 4–1).

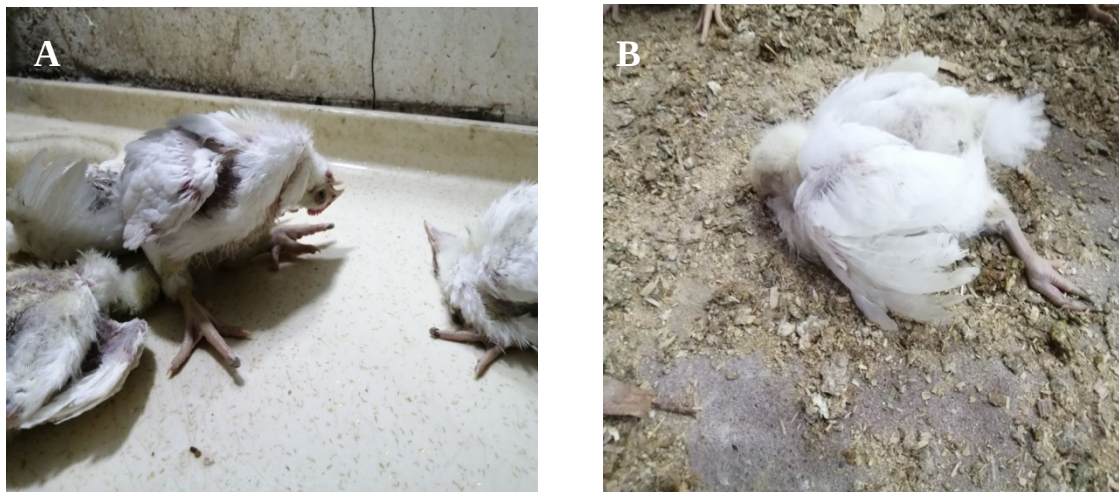


Fig.(4-1) Clinical signs in infected birds with NDV (A) paralysis of legs and wings, twisting of neck due to nervous system infection : (B) respiratory signs and paralysis.

4.2.2. Gross lesions in post mortem examination

During postmortem examination of dead birds or birds with severe clinical signs, necrotic lesions were observed in the mucosa of the intestine, proventriculus, and gizzard (Fig. 4-2 A, B). Furthermore, hemorrhagic lesions were very prominent in the mucosa of the proventriculus (Fig. 4-2A). Regarding the PM symptoms related to the respiratory system, it was seen that air sacs were filled with whitish translucent material, but the lungs were normal in size. Also, enlarged mottled spleen and congested enlarged liver were also observed (Fig. 4-2 C, D).

Additionally, dehydration of carcass with muscle congestion, cecal hemorrhage and pin-point hemorrhages on tips of the proventricular glands, greenish intestinal contents, and hemorrhagic mucosa of the trachea are also seen in early-dead birds.



Fig.(4-2) Postmortem symptom observed in the body of infected birds with NDV (A) proventriculus haemorrhage (B) cecal tonsils haemorrhage (C) haemorrhage and congestion of intestine (D) enlargement and congestion of liver and spleen.

4.3. Immunological detection

4.3.1. NDV Immunochromatographic rapid assay

A field examination was performed by an NDV rapid kit, which showed positive results for 52 (62.65%) samples out of 83 that were detected as ND according to clinical symptoms. The positive results were read by the appearance of the indicative line at the letter T (test) and the letter C (control) together (Fig. 3), while the negative result was at the C (control) line.

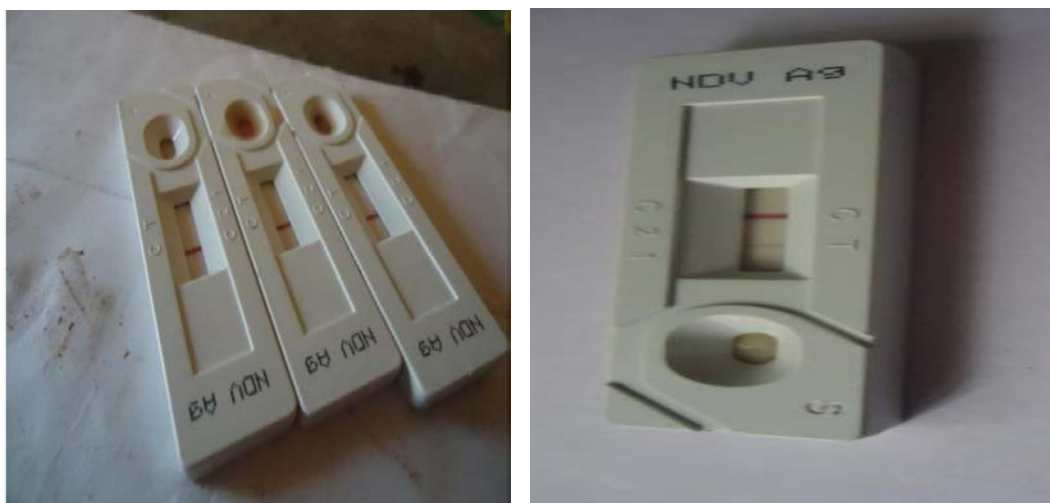


Fig.(4-3) Kit showing purple color band at both T & C lines (positive), Kit showing no purple color band at T line (negative).

4.4. Molecular detection of ND virus in the samples

The matrix (M) gene of NDV was amplified successfully and detected in about 27 samples out of 52 samples. However, the Ct values vary by sample from 18 to 31 depending on the concentration of the NDV in the samples, reflecting the intensity of the infection (Fig. 4-4A). The low Ct values and high NDV concentration were reported in only 8 samples. Also, using

conventional PCR for the F gene (565 bp), only 6 samples showed strong bands located at about 550bp and 400 bp, respectively (Fig. 4-4 B).

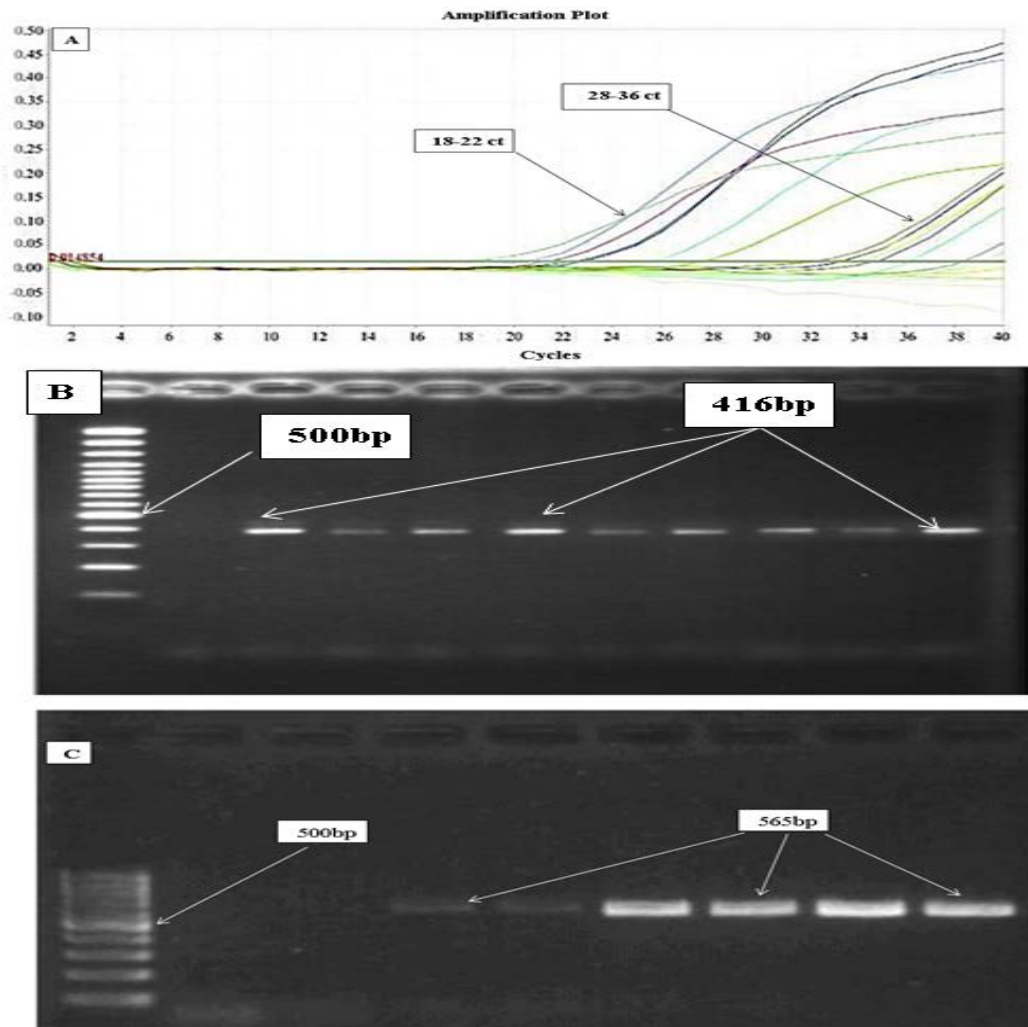


Fig.(4-4) A. Amplification plot for RT-PCR showing the Ct value for matrix gene ; B and C are conventional PCR for HN and F gene products validated by 2% agarose gel electrophoresis, respectively.

4.5. Gene expression data of inflammatory biomarkers

For the eight positive samples detected by RT-qPCR, several markers have been tested to observe the inflammatory response induced by NDV in infected birds compared with healthy birds. Specifically, CRP gene expression was showed a significant increase in birds after two days (48 h) of infection, documented by about twofold changes (Fig. 4–5). In contrast, IL6, MMP9, Il1 β , and Inf γ mRNA expression increased significantly after 24 hours of infection and showed 3–4 fold changes in mRNA expression compared with their expression in non-infected birds (Fig. 4–5).

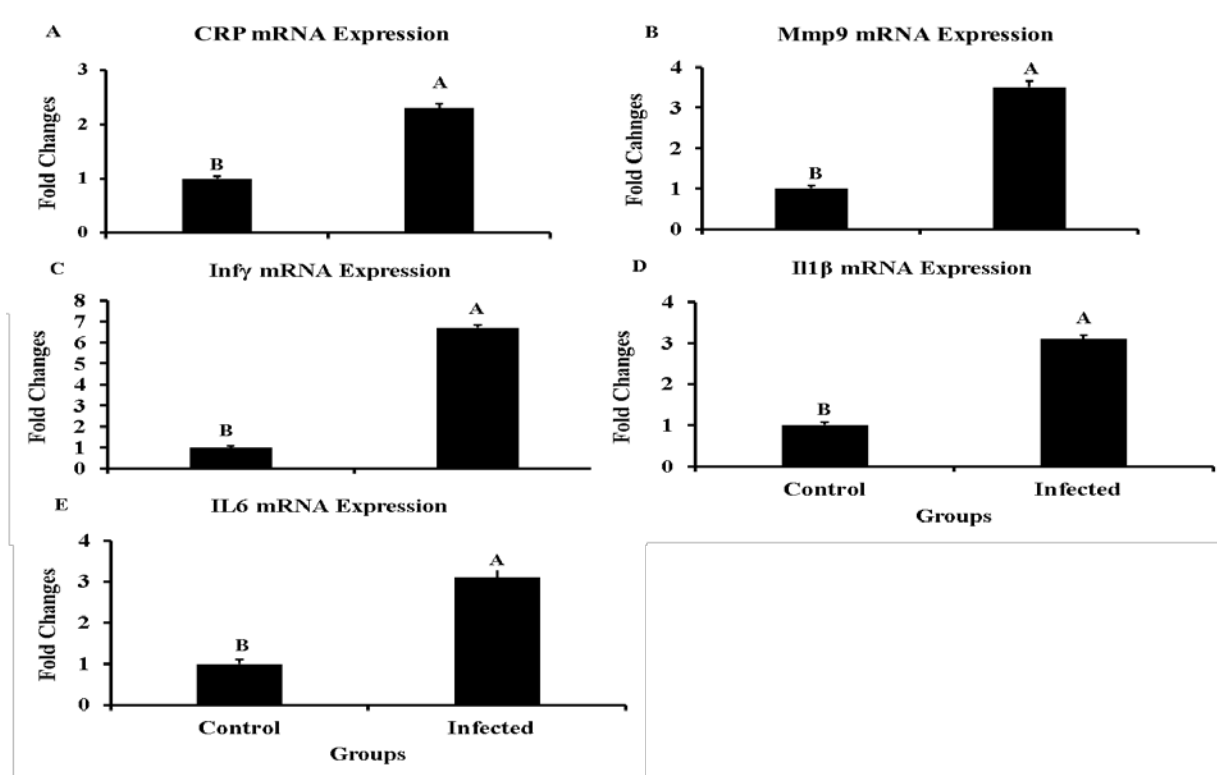


Fig.(4-5) Fold changes in the mRNA levels for the inflammatory biomarkers in the infected birds comparing with non-infected birds : A=CRP, B=Mmp9, C=Infy, D=IL1 β , and E=IL6.

4.6. DNA sequencing of selected region of gene F of NDV

4.6.1. FASTA files isolates (Sequenced by Macrogen, Korea) and alignment of the ND isolates based on the sequences of F gene

Fasta, alignment, and phylogenetic tree of the nucleotide sequences of F gene of the pooled samples (AK) against the ND virus sequences in the NCBI database were presented (Fig.4-6). Results showed that the isolate has about 88.27% similarity with avian orthoavulavirus-1 isolate Ph/IR/AMMM116/2018 fusion protein gene.

>AK_F gene Fasta

```
CCCAGGGTTAAGGACGGGTGTTTCGAGCAAACAATCTGAGGCAAA
TATTGGAACACTGACTGCTTTGCCCACTCCTCTTGGTGACTCAAA
CCGGAAGATCCAAAAGTCTGTGTTCACATCTGGAAAGGGGAGGC
AAAAACTGTTTATAGTGCGTGTTATTGGCAGTCGAGCTCTTGGGG
TTGCAACAGCGGCACAGATAACAAGCTGCGCAGCCTAATACAAG
CCAACCAAAAATGCCGCCAACATCCTCCGAGCCTTAAGGAGAGGT
TGCTGCAACCAATGAAGCTGTGCATGAAGTCCCAAACGGATTATC
ACAACATA
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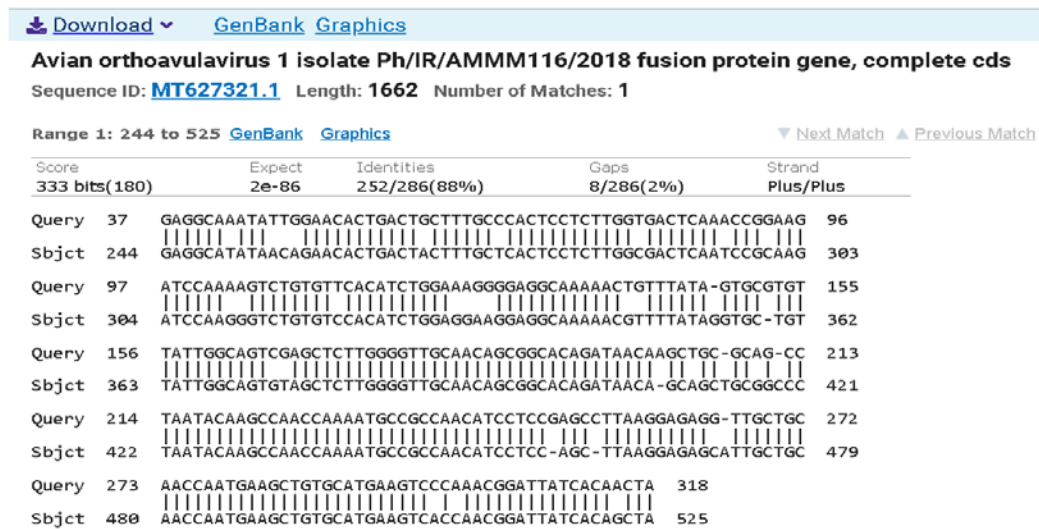


Fig.(4-6), Alignment of fasta data for F gene against avian orthoavulavirus 1

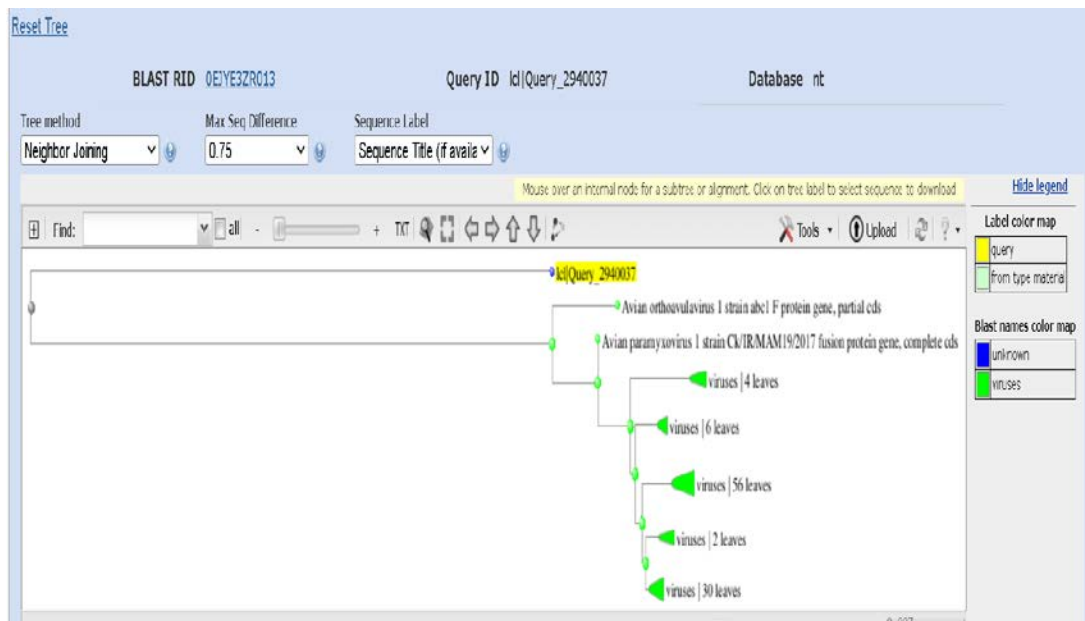


Fig.(4-7), Phylogenetic tree for F gene against sequences available in the NCBI

Chapter Five

Discussion

5. Discussion

5.1. ND induces a variety of clinical manifestations and post mortem lesions

The birds infected with NDV showed different clinical signs ranging from mild to severe signs. Specifically, the main manifestations observed in the infected cases were coughing, weakness, sneezing, gasping for air and nasal discharge (Fig.4-1 B). Regarding digestive signs, the main signs observed in the infected cases were the greenish diarrhea. Furthermore, paralysis of wings and/or legs was a nervous sign. Also, twisting of the head and neck as well as whole body paralysis were observed in many cases (Fig.4-1 A). These signs could be attributed for the virulence of the virus and its ability to infect different body system.

The respiratory signs, such as sneezing, coughing, gasping and nasal discharge maybe due to ND infection in bronchi or pneumonia. These signs have been reported by other researchers who observed the same signs in chicks infected with ND (Getabalew *et al.*, 2019). Consistent with our results in terms of digestive signs, studies showed that greenish watery stool can be a sign of digestive complications such as malabsorption syndrome, dehydration and malnutrition (Bhutia *et al.*, 2017). Furthermore, the nervous signs observed were paralysis and head twisting due to the ability of the virus to infect central nervous system. During ND infection, it has been shown that when virulent NDV strains infect central nervous system (CNS), viruses can replicate in neurons causing various degrees of non-supportive encephalitis and severe neurological signs. The

findings in the current study agreed with findings of other researchers who discussed that previously (Cattoli *et al.*, 2011; Ecco *et al.*, 2011).

In the postmortem examination of dead or severely infected birds, necrotic and hemorrhagic lesions were observed in the mucosa of intestine, proventriculus and gizzard (Fig. 4-2 A,B and C). Also, enlarged and mottled spleen, congested and enlarged liver were also seen in Fig. 4-2D. Other lesions were also detected in the infected and/or recently dead birds such as dehydrated and congested muscles, dehydrated carcass, periventricular gland-tips hemorrhages, and greenish intestinal contents. This could be due to loss of appetite and inability of infected birds to reach to the food and water as well as the high temperature resulted from viral infection. Additionally, the P.M. symptoms related to the respiratory system were that air sacs filled with whitish translucent material and catarrhal tracheitis with hemorrhagic mucosa. However, lungs were normal in size.

The possible explanation for gastrointestinal haemorrhage was that digestive system organs were infected with high concentrations of the virus, as they are a favorable environment for its growth, which leads to severe damage to those organs. The P.M observations in the study were observed by other groups of researchers (Mohammed *et al.*, 2013; Sen *et al.*, 2017; Awad *et al.*, 2020). Chickens infected with NDV that had just died were found to have hemorrhagic ulcers in the intestinal walls, enlarged spleens, degeneration and multifocal necrosis in their livers, and pinpoint hemorrhages in their proventriculi during postmortem inspection. Furthermore, a highly affected spleen will be larger and heavily mottled,

with many areas of white to yellow discoloration (necrosis) (Cattoli *et al.*, 2011). Moreover, dehydrated carcass as well as dehydrated and congested muscles could be due to the high temperature in the body of bird, which may be caused by the immune response to the virus. Respiratory system infections could lead to the death of the bird due to suffocation and lack of oxygen supply to the body's organs. Therefore, the possibility of secondary infections with the NDV remains somewhat possible, for example pneumonia, bronchial congestion, and laryngitis (Awad *et al.*, 2020).

5.2. ND virus changes mRNA levels of inflammatory biomarkers

In reviewing the literature, a few papers addressed the mRNA expression of inflammatory biomarkers or cytokines in chickens from ND-infected birds. Therefore, in the current study, RT-qPCR was utilized for the positive samples, and several markers were tested to observe the inflammatory response induced by NDV in infected birds compared with healthy birds. Specifically, C-reactive protein (CRP) gene expression showed a significant increase in birds after two days (48 h) of infection, resulting in about twofold changes (Fig.4-5).

In contrast, IL6, MMP9, IL1 β , and Inf γ mRNA expression increased significantly after 24 h of infection and showed 3-4 fold changes in mRNA expression compared with their expression in non-infected birds (Fig. 4-5). In this study, it was observed that the level of CRP in the liver increased more than the control data (Fig. 4-5 A). A high CRP level indicates that there is inflammation somewhere in the body of birds. This could be due to viral exposure and suggests an infection with the ND virus. Thus, ND infection seems to be responsible for that rise. The birds positive for

E.coli, *Pasturella multocida* and *Staphylococcus aureus* infection as well as histomoniasis and adjuvant injection found to be positive for CRP. These birds on post mortem had lesions consistent with chronic respiratory disease, which is highlighting the use of CRP as potential biomarker for non-clinical disease. (Sohail *et al.*, 2010)

The second biomarker tested was matrix metalloproteinase-9 (MMP-9). The above biomarker, MMP9, has an elevated level (Fig. 4-5B). The high levels of MMP-9 in the bodies of birds could indicate that the body is working overtime to repair or remodel tissues, which is often a response to inflammation or tissue damage. This rising can be seen in a variety of health conditions. For instance, in the heart and lung diseases can also trigger an increase in MMP-9 as the body tries to repair inflamed tissues. However, it is normal to find a high MMP-9 level in the serum of infected birds due to the direct effect of the NDV on the lungs and heart (Nandi *et al.*, 2020).

Furthermore, the third biomarker measured in this study was interferon gamma (IFN- γ or IFNG). The IFN- γ gene expression increased significantly in the infected birds compared with controls (Fig. 4-5 C). It is known that IFN- γ is a cytokine secreted by immune cells (i.e., NK cells and T lymphocytes).

IFN- γ and its adjuvant characteristics have potential antiviral activity against avian viral pathogens such as avian influenza virus (AIV), NDV, and Marek's disease virus (MDV) (Sawant *et al.*, 2011; Susta *et al.*, 2013; Haq *et al.*, 2015). Thus, the possible increase of IFN- γ in the current study is conclusive evidence of the immune response to viral infection with NDV.

The fourth biomarker measured in the infected birds and compared with controls (non-infected) was interleukin-1 beta (IL-1 β). Although elevated IL-1 β expression is common with NDV infection (Fig. 4-5 D), it is not unique to this virus, as several others, including influenza A virus, infectious bronchitis virus, Sendai virus, and vesicular stomatitis virus, also increase IL-1 β expression, as previously documented in other studies (Thomas *et al.*, 2009; Wang *et al.*, 2016; Thi and Hong, 2017; and Amarasinghe *et al.*, 2018). In fact, a study on influenza A virus found that following inhibition of the IL-1 β receptor, lung pathology caused by the influenza virus was significantly reduced, indicating that IL-1 β is a principal component associated with pulmonary inflammation during H1N1 influenza viral infection (Kim *et al.*, 2015).

The last biomarker tested in the infected samples was interleukin-6 (IL-6). IL-6 mRNA expression was high in the infected birds compared with the control (Fig. 4-5 E). IL-6 is implicated in the host of chronic disease conditions associated with inflammation. Queiroz and his colleagues reported that IL-6 is one of the important cytokines, which is relevant to the outcome of COVID-19, including disease duration and severity (Queiroz *et al.*, 2022). IL-6 is generated at sites of infection and inflammation by immune cells (Tanaka *et al.*, 2014), adipocytes (Fain, 2010), and endothelial cells (Chi *et al.*, 2001).

The cytokine promotes the differentiation of naive CD4⁺ T cells, which suggested that IL-6 is an essential factor in the development of adaptive immunity (Tanaka *et al.*, 2014). Therefore, the high temperature resulted from viral infections and severe inflammation that may occur due to the same infection with ND. It is likely that the increase is the result of a high

immune response and the production of more IL-6. The findings of this study are consistent with other studies that addressed proinflammatory cytokine profiling and found that NDV predominantly increased the production of interleukin 6 (IL-6) in most cells (Chhabra *et al.*, 2018; Balkrishna *et al.*, 2022).

Furthermore, another group of researchers found that IL-6 was highly expressed. IL-6 has been linked to the inflammatory response to viral infection in several cell lines, and its production is induced by activation of nuclear factor kappa B (NFB) (Jia *et al.*, 2018). However, it is essential to determine whether IL-6 aids in tumor selectivity and oncolysis.

5.3. Molecular changes in the ND virus

To explain the sudden appearance of highly pathogenic NDV, Madadgar *et al.* (2013) proposed three hypotheses: (1) the virus was always in poultry but was unnoticed until the advent of commercial poultry industries; (2) the virulent virus was enzootic in another species where the less sensitive host revealed the less severe disease; and (3) the highly pathogenic virus arose from a virus of low virulence by mutation.

Therefore, a molecular approach, PCR, was applied to identify genetic variations in the ND virus to explain ND outbreaks in Iraq despite regular and intensive immunization. Moreover, it has been proposed that RT-PCR be employed due to its high sensitivity, high specificity, efficiency, and especially its ability to identify the ND virus (Selim *et al.*, 2022). RT-PCR was utilized for positive samples detected by the rapid test. Three genes, which are the matrix (M) gene, the haemagglutination (HN) gene, and the fusion (F) gene were amplified successfully by PCR. This result was consistent with the findings of Alsahami *et al.* (2018), who isolated and

identified the NDV from suspected samples using reverse transcriptase real-time PCR.

In the current study, the M gene, which is a standard gene used for detection of NDV infection with high sensitivity, was observed in about 27 samples out of 52 positive samples detected by the NDV rapid test. The possible explanation is due to the low concentration of viral nucleic acid in the tested sample, and this could be supported by the Ct values recorded in the results. In the current study, M gene Ct values differ from sample to sample, range from 18 to 31, and appear to be dependent on the concentration of NDV in the samples, reflecting the intensity of the infection (Fig. 4-4A).

Ct value variations indicated that the concentrations of viruses in the sample are not the same among samples. Furthermore, this variation was due to the fact that the total RNA was extracted from each sample, not just viral RNA. Similarly, previous studies showed that 26 out of 34 fields were positive when tested by RT-PCR (Hasan *et al.*, 2010). Additionally, another group of researchers reported in hens that NDV was detected in 17 out of 63 samples in the lungs by using reverse transcriptase real-time PCR (Worku *et al.*, 2022).

Unlike the findings in this study, Ahmed and Odisho (2018) showed that all the tested samples were positive for NDV when their RNA was analyzed using reverse transcriptase-polymerase chain reaction (RT-PCR). This could be attributed to the primers used in the study or other strains in the area. Therefore, this conclusion should be considered.

Thereafter, conventional PCR was applied to positive samples to amplify the F gene. Regarding the nucleotide sequences of the F gene in

the current study, fasta, alignment, and the phylogenetic tree of the nucleotide sequence data displayed that the isolate has about 88.27% similarity with the avian orthoavulavirus 1 isolate Ph/IR/AMMM116/2018 fusion protein gene.

Data indicate that possible outbreaks in Iraq may be caused by variations in the nucleic acid of field strains in Iraq from that in the vaccine strain. So, an alignment with reference strains is needed including vaccine strains. Similarly, there have been substantial updates to the genomic data of NDV in the databases. Notably, the F gene primers used in the current study may not work perfectly to match the sequences of all APMV-1 isolates in the databases at the time (Wise *et al.*, 2004).

The program MEGA11 was used for the evolutionary analyses (Kumar *et al.*, 2016). Neighbor-joining was used to estimate the evolutionary history (Saitou and Nei, 1987). The taxonomic lineage is assumed to follow the tree of life derived using the bootstrap method (500 replicates) (Felsenstein, 1985). When a partition is repeated in less than half of the bootstrap replicates, the relevant branch is pruned. In the bootstrap test (500 repetitions), the proportion of trees whose connected isolates is grouped together. It is shown next to the branches (Felsenstein, 1985).

Conclusion

Conclusion

Conclusion

- 1- This study addressed gene expression to observe changes in the mRNA levels of several inflammatory biomarkers.
- 2- The data is showed an increase in all the inflammatory biomarker mRNA levels in infected birds compared with controls.
- 3- Utilizing the molecular approach, PCR, to sequence the F gene displayed a deviation from the available sequences in the NCBI.
- 4- Data indicated that the recurrence of ND infection in the vaccinated flocks was due to a mismatch between the vaccine sequence and the infected strains.

Recommendations

Recommendations

Recommendations

- 1- Research priorities should focus on improving diagnostic tools and developing better vaccines.
- 2- Any control approach should focus on the appropriate characterization of the virus strain causing the outbreak in the study area.
- 3- Produce a high-quality and up-to-date vaccines from the isolated strains or use a vaccine that has less genetic variance with endemic strains.

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الخلاصة

صممت هذه الدراسة للتعرف على النمط المصلي المتوطن لفيروس مرض نيوكاسل الناتج عن تفشي المرض الشديد في محافظة ذي قار والذي يتميز بارتفاع معدل الهلاكات والأعراض التنفسية والهضمية والعصبية.

اعتماداً على تاريخ الحالة والعلامات السريرية وتشريح الجثة، تم جمع 83 عينة (مشتببه باصابتها) و40 عينة من طيور سليمة وعدت كمجموعة سيطرة للطيور بعمر من 20 الى 35 يوماً خلال الفترة من أكتوبر 2023 إلى فبراير 2024. في بداية هذه الدراسة، تم تأكيد الإصابة باستخدام الاختبار التشخيصي السريع للكشف عن مستضدات فيروس نيوكاسل. تم جمع الدم، الكبد، القسبة الهوائية، الرئة، والأعور في أنبوب سعة 1.5 مل يحتوي على حوالي 1 مل ترازول لاستخراج الحمض النووي الريبي (RNA). كما تم عزل المصل من عينات الدم وتجميده عند درجة حرارة (-20 مئوية). تم تجميد جميع العينات الأخرى في النيتروجين السائل (-196 درجة مئوية).

خضعت جميع العينات التي اظهرت نتيجة ايجابية باستخدام الاختبار التشخيصي السريع الى الاختبارات الجزيئية بعد استخراج الحمض النووي الريبي (RNA). لذلك، تم استخراج إجمالي الحمض النووي الريبي (RNA) وتنقيته وقياسه كماً باستخدام مقياس الطيف الضوئي النانوي. بعد ذلك، تم تحويل الحمض النووي الريبي (RNA) إلى الحمض النووي المكمل (cDNA) الذي تعرض لتقنيات تفاعل البوليميريز المتسلسل في الوقت الحقيقي (rt-PCR) للكشف عن الحمض النووي لفيروس نيوكاسل في العينات، وتم استخدام تفاعل سلسلة البوليميريز العكسي (RT-PCR) لهذا الغرض. أيضاً، تم استخدام PCR التقليدي للجين F (356 زوج من القواعد) والجين HN (416 زوج من القواعد).

على الرغم من أن العديد من الدراسات السابقة تستخدم الاختبارات المصلية لتحديد كمية الأجسام المضادة للمؤشرات الحيوية الالتهابية في عينات المصل وأظهرت انخفاض الأجسام المضادة بشكل كبير في حالات العدوى الفيروسية. في هذه الدراسة، تم اختبار العديد من علامات الالتهاب الحيوي في العينات الإيجابية التي اكتشفها RT-qPCR، لمراقبة الاستجابة الالتهابية الناجمة عن فيروس نيوكاسل في الطيور المصابة مقارنة بالطيور السليمة. على وجه التحديد، أظهر التعبير الجيني لبروتين C التفاعلي (CRP) زيادة كبيرة في الطيور بعد يومين (48 ساعة) من الإصابة موثقة

بحوالي ضعفي التغييرات. في المقابل، زاد تعبير mRNA للإنترلوكين 6 (IL-6)، وماتركس ميتالوبروتيناز 9 (MMP-9)، والإنترلوكين 1 بيتا (IL-1 β)، وإنترفيرون جاما (Inf- γ) بشكل ملحوظ بعد 24 من العدوى التي أظهرت تغيرات بمقدار 3 إلى 4 أضعاف في تعبير mRNA مقارنة مع ظهورها في الطيور غير السليمة. لذلك فإن ارتفاع درجة الحرارة ناتج عن التهابات فيروسية وحدث التهاب حاد بسبب نفس الإصابة بـ ND قد يكون نتيجة لارتفاع الاستجابة المناعية. علاوة على ذلك، تم تطبيق النهج الجزيئي، PCR، لتحديد الاختلافات الجينية في فيروس ND لتفسير تفشي مرض ND في محافظة ذي قار على الرغم من التحصين المنتظم والمكثف.

أظهرت نتائج تحليل بيانات تسلسل النيوكليوتيدات لبروتين F تشابهًا بنسبة 88.27% و89.44% مقارنة بالعزلات الموجودة في البنك الجيني (NCBI). ختامًا، إن تفسير تكرار الإصابة بفيروس نيوكاسل في محافظة ذي قار على الرغم من أن برنامج التحصين المكثف مع ارتفاع معدلات الهلاكات. ولذلك، يلزم استخدام لقاح ذو تشابه كبير مع سلالات فيروس نيوكاسل المستوطنة في المحافظة.



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فرع الاحياء المجهرية

المؤشرات الحيوية الالتهابية للدجاج اللاحم المصاب بفيروس مرض نيوكاسل المنتشر مؤخراً في محافظة ذي قار

رسالة مقدمة الى مجلس كلية الطب البيطري, جامعة ذي قار وهي جزء من متطلبات
نيل درجة الماجستير في الطب البيطري /الأحياء المجهرية

من قبل

عباس كامل شلاكه

بكالوريوس طب وجراحة بيطرية

كلية الطب البيطري / جامعة واسط (٢٠١٦)

بإشراف

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