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Expression of Newcastle disease virus genes and inflammatory biomarkers in Newcastle disease infected chicks

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Abstract

In poultry, the devastating effect of Newcastle disease (ND) has been addressed; however, the inflammatory response of infected birds has received less attention. Therefore, the study was designed to detect the ND virus and find changes in the gene expression of inflammatory biomarkers in birds infected naturally with ND using reverse transcription-PCR (RT-PCR). The expression of the matrix (M) gene of NDV and inflammatory biomarkers like creative reactive protein (CRP), interleukin 6, interleukin-1 beta (IL-6, IL-1β), and gamma interferon (IFN-γ) genes was analyzed. Despite the fact that flocks were vaccinated against NDV using the LaSota vaccine, various clinical signs were observed in many flocks, and 60 suspected and 30 control samples were collected from 3 to 5 weeks old. Blood, liver, and tracheal swaps were sampled from suspected and control birds. However, the infected birds showed respiratory, digestive, and nervous signs. Further, typical postmortem symptoms such as mottled spleen, periventricular gland-tip hemorrhages, and cecal hemorrhages were seen. The NDV infection was initially confirmed using the NDV rapid test, which showed positive results in 52 out of 60 suspected samples (86.66%). To confirm the infection with NDV, RT-PCR was utilized to examine the expression of the M gene in the samples and revealed that only 27 (51.92%) out of 52 samples were positive. Thereafter, the mean antibody levels of ND-infected birds were significantly lower than those of uninfected birds. In contrast, inflammatory biomarkers' gene expression exhibited an increase in their mRNA levels, indicating that the birds were infected with a viral infection and that there is inflammation in the body. To sum up, data suggest that the M gene could be used as a marker for identifying NDV in infected birds. Similarly, the NDV infection leads to a decrease in AB titers, which is associated with an increase in the gene expression of inflammatory biomarkers in infected birds. Therefore, serological tests as well as the molecular approach should always be considered in epidemic regions.

Keywords: Matrix gene, inflammatory biomarkers, RT-PCR

Introduction

One of the worst illnesses affecting chickens worldwide is Newcastle disease (ND) (Shabbir et al., 2013). Although depression, neurological symptoms, or diarrhea may be the most common clinical form, it is a global issue that typically manifests as an acute respiratory illness. Furthermore, the high rates of mortality in the ND infection limit the growth of poultry production. Additionally, four ND epidemics that were caused by distinct NDV genotypes have been recorded globally, which affected global trade seriously (Diel et al., 2012). Turkeys, chickens, and other poultry are susceptible to ND, which is a deadly, contagious disease, and it can be categorized based on pathogenicity into velognic, mesogmic, or lentogenic. NDV is an enveloped virus with a negative polarity, non-segmented, single-stranded RNA. It has a matrix (M) protein on the internal surface of the envelop (Miller and Emmerson, 1988). NDV infects the host cells of targeted species in different ways, starting with virus entry via haemaggluatination neuraminidase (HN) and fusion (F) glycoproteins. The HN interacts with the host

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cell surface via cell receptors that are composed of sialic acid. On the other hand, viral entry is attributed to the F protein. Following fusion, viral nucleic acid (nucleocaspid) enters the host cell cytoplasm, where transcription and translation phases start (Mao et al., 2022). Furthermore, cytokines act on target cells by attaching to specific receptors and can enhance or inhibit cell functions. Interleukin, tumor necrosis factor, and chemokines are types of cytokines classified based on their function and synthesis location. On the other hand, there is a lot of crossover across the various classifications (Mitra and Leonard, 2018). Gamma interferon (IFNγ), interleukin 6 (IL-6), and interleukin 1 beta (IL-1β) are multifunctional cytokines that have essential roles in acute-phase responses, immunological control, and haematopoesis, and are released by many cells. Serum amyloid A and C reactive protein (CRP) are examples of acute-phase proteins that are produced as part of an inflammatory response to infection or other stressors in animals (Aliyu et al., 2022). Furthermore, despite the development in the laboratory diagnostic tools, diagnosis still relies mainly on the field examination and serological tests. Therefore, we hypothesized that besides serological tests such as HI and antigen rapid tests, RT-PCR would be an essential molecular method to identify ND infection in each province. To conduct our hypothesis, serological tests such as antigen rapid and HI tests will be used to detect a possible NDV infection; then, matrix (M) gene expression will be measured by RT-PCR as an indicator of NDV infection. Further, antibody titers in the serum will be measured for the infected and control birds. Similarly, the inflammatory response will be evaluated by studying the mRNA levels of inflammatory biomarkers, which are CRP, IL-6, and IL-1β, as well as interferon gamma.

I. Materials and Methods

Based on the case history, clinical signs, and postmortem symptoms, samples were collected from 3-5-week-old broilers. The number of birds was 30 samples (control) and 60 samples (suspected cases). The initial diagnosis was performed using the NDV rapid kit (Bionote, Korea). Samples of blood, liver, and tracheal swabs were gathered and snap frozen rapidly, except for the blood samples. The blood sample was divided into two part; one for serological and the second (200 µl) was mixed with Trizol for RNA extraction using the Trizol-Chloroform protocol (Kadhim et al., 2020). While the second half was assigned for measuring antibody titer in the infected and control birds using HI test (Reda and Jasim 2022).

For the positive samples detected by the NDV Rapid test, viral Gene-spin^{TM}Viral DNA/RNA extraction Kit was used to extract viral RNA from tracheal swaps for matrix (M) gene expression to confirm the ND infection. While total RNA was extracted from blood and liver samples for inflammatory biomarkers' expression using Trizol-Chloroform, and RNeasy mini kit (Qiagen) was utilized to purify RNA (Kadhim et al., 2019). For viral and total RNA, Dnase1 was used to remove genomic DNA. Then, concentration of RNA was measured using nanodrop spectrophotometry. Thereafter, Superscript®III (Invitrogen) was used for cDNA formation as described in our previous publications (Kadhim et al., 2022; 2021). In RT-PCR, samples were achieved using Power SYBR Green Master Mix in 30 µl and ran in duplicate for 40 cycles. Inflammatory biomarkers, CRP, IL-6, IL-1β, and IFN-γ, were measured, and glyceraldehyde3-phophate dehydrogenase (GAPDH), was a housekeeping gene. The primer details were described (Table 1) Fold changes in mRNA expression were calculated for inflammatory biomarkers after normalization with a housekeeping gene using the $2^{-\Delta\Delta Ct}$ equation (Schmittgen and Livak, 2008).

Statistical Analysis

JMP® 18.0 was used to analyze gene expression and antibody titer data. A student t-test and Tukey's HSD test were calculated to evaluate the significant effects of infection and compared with control. Fold changes in the mRNA levels were calculated and presented as mean ± SEM, with $p \leq 0.05$ considered significant statistically.

II. Results

Clinical signs and postmortem symptoms: Based on field observation, the infected birds showed a variety of clinical signs, such as respiratory, nervous, and digestive signs. The main clinical signs were decreased appetite, coughing, watery eyes, nasal discharge, greenish watery diarrhea, and nervous manifestations, which were head and neck twist, legs and/or wings paralysis, or whole body

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paralysis (Fig. 1A and B). During postmortem examination of birds with clinical signs, spots of necrosis were observed in the gizzard, proventriculus, and intestine (Fig. 1C and D). Furthermore, hemorrhage spots in the proventriculus were widely found. Also, mottled spleen and an enlarged liver were observed (Fig. 1E and F). In the early dead birds, several other symptoms were observed, such as dehydrated congested muscles, dehydrated carcass, periventricular gland-tip hemorrhages, congested and hemorrhage of the intestine, and catarrhal tracheitis.

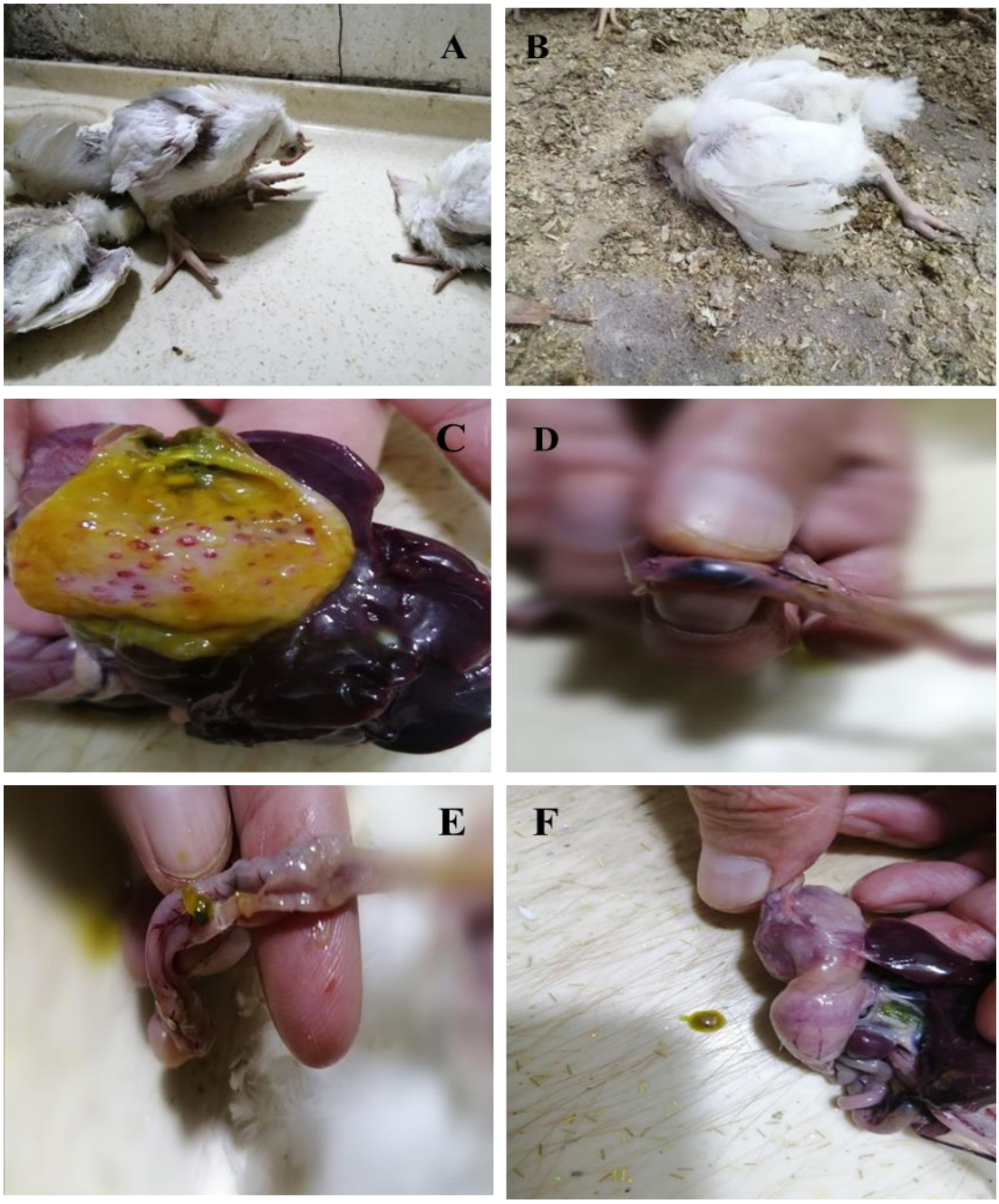

Fig. 1. Clinical signs and postmortem symptoms of infected birds with NDV (A) paralysis of legs and wings, twisting of neck due to nervous system infection; (B) respiratory signs and paralysis; (C) proventriculus haemorrhage (D) cecal haemorrhage (D) haemorrhage and congestion of intestine (F) enlargement and congestion of liver and spleen.

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NDV rapid test: The test was performed in the fields for suspected cases (with clinical signs), the results of the test showed that 52 (86.65%) samples out of 60 were positive, as evidenced by the two lines appearing on the device.

Haemaggluatination inhibition (HI) test: Utilizing the HI test to measure AB titers in the infected and control birds, the AB titers decreased significantly in the infected birds compared with (Fig. 2, $p < 0.001$; T-test = 11.32). The mean AB titer in the control birds was (532.15±32.12), while it was (34.27±2.32) in the infected birds.

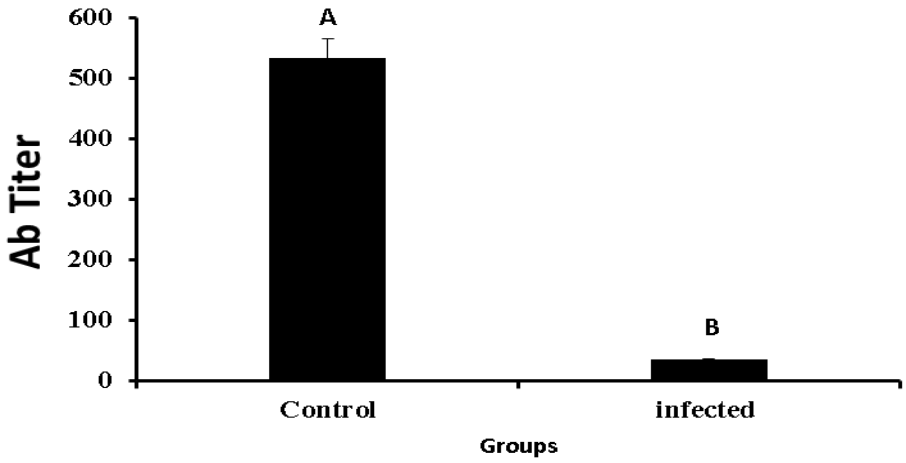

Figure 2. Antibody titer infected birds and control group.

Molecular Diagnosis for NDV: Utilizing reverse transcription-PCR (Fig.3), the matrix (M) gene, which is a universal protein for NDV detection, was successfully amplified and detected in 27 (51.92%) samples out of 52; however, the ct values vary by sample from 18 to 33 cycles.

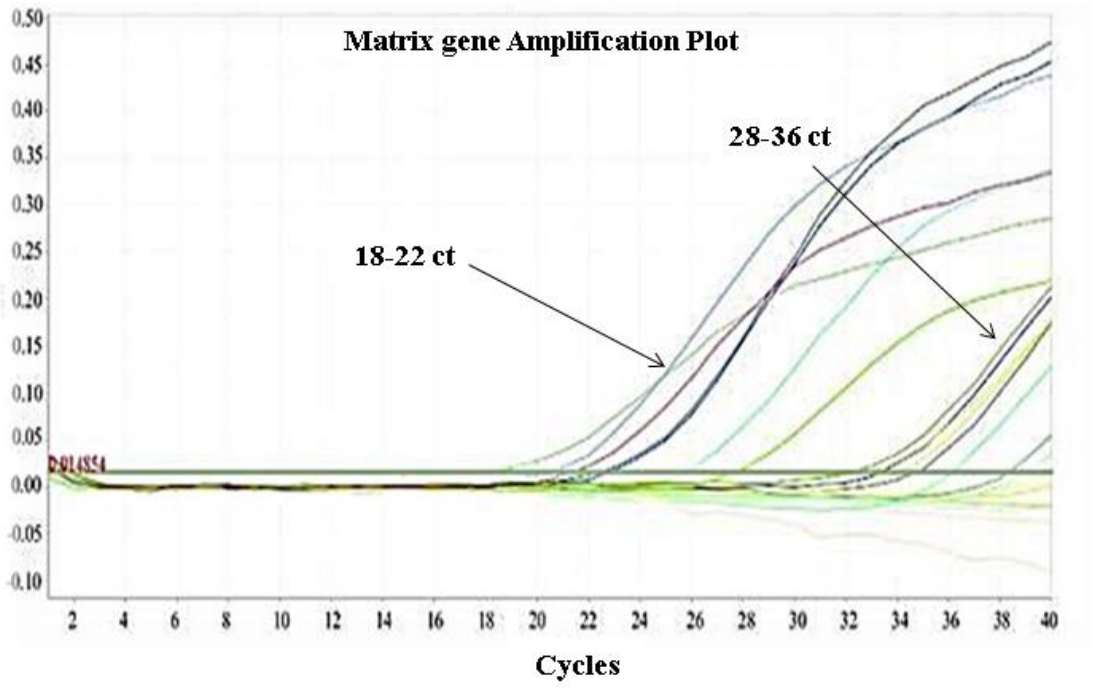

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Figure 3. Amplification plot for matrix gene of Newcastle disease virus by RT-PCR in naturally infected chiks.

Gene expression data of inflammatory biomarkers: several biomarkers were tested to observe the inflammatory response induced by NDV in infected birds compared with healthy birds (Fig. 4). Specifically, CRP gene expression showed a significant increase in birds after two days (48 h) of infection, documented by about twofold changes ($p<0.01$). In contrast, IL6, Il1β, and Infγ 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. Remarkably, the Infγ mRNA expression showed more than a six-fold increase ($p <0.001$).

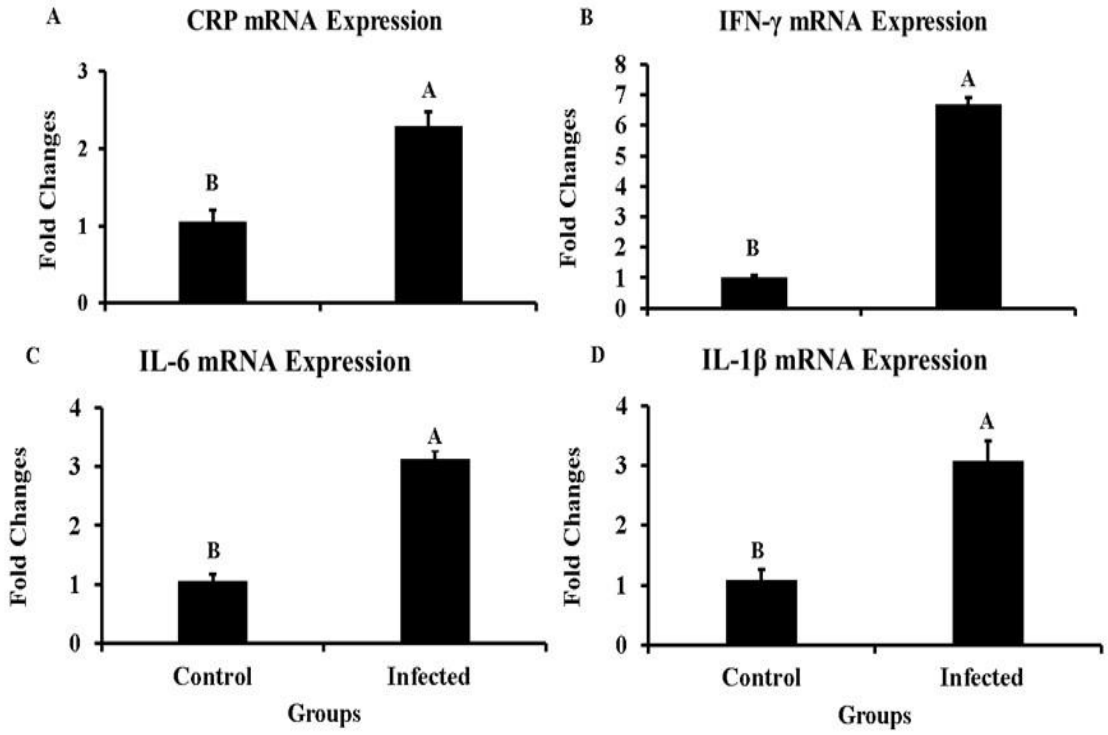

Figure 4. Changes in the mRNA levels for the inflammatory biomarkers in the infected birds comparing with non-infected birds : A=CRP, B=IFN-γ, C=IL6 and D=IL-1β.

Discussion

The researchers utilized RT-PCR to detect NDV and identify changes in mRNA expression of inflammatory biomarkers in infected flocks. The birds exhibited a variety of signs, including respiratory, digestive, and nervous (Fig.1). In this context, Getabalew et al. (2019) reported that the respiratory signs may be due to an ND infection in the bronchi or pneumonia. Furthermore, the greenish, watery stool can be a sign of digestive complications such as malabsorption syndrome, dehydration, and malnutrition (Bhutia et al., 2017). During ND infection, when virulent NDV strains infect the central nervous system (CNS), viruses can replicate in neurons, causing encephalitis and nervous signs, as discussed previously by Cattoli et al., (2011) and Ecco et al. (2011). Moreover, postmortem examination revealed typical symptoms such as mottled spleen, congested and enlarged liver, periventricular gland-tip hemorrhages, and catarrhal tracheitis (Fig. 1) Similarly, other researchers reported the same P.M. symptoms (Sen et al., 2017; Awad et al., 2020). Notably, utilizing NDV rapid test exhibited that not all cases with clinical signs were positive (86.65%). As we know, the principle of the NDV rapid test kit depends on antibody-

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antigen reaction. Therefore, the possible explanations for that are test sensitivity, antibody-antigen compatibility, and/or other viral infections. Similarly, the antibody titer decreased significantly in the infected birds (Fig. 2).

To detect ND infection in chicks suffered from above clinical signs and P.M. symptoms, a molecular approach, PCR, was applied, which is the most specific and efficient approach for detecting NDV is the RT-PCR (Selim et al., 2022). RT-PCR was utilized for positive samples detected by the rapid test. The matrix (M) gene was amplified successfully by PCR (Fig.3). Similarly, Alsahami et al. (2018) identified the NDV in the suspected cases by RT-PCR. In this study, the M gene, which is a standard gene used for detection of NDV infection with high sensitivity, was observed in about 27 samples (51.92%) 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 33, and appear to be dependent on the concentration of NDV in the samples, reflecting the intensity of the infection. Ct value variations indicate that the concentrations of viruses in the sample are not the same among samples. Similarly, previous studies showed that 26 out of 34 fields were positive when tested by RT-PCR (Hasan et al., 2010). Utilizing RT-PCR for lung samples, the NDV was detected in 17 out of 63 samples (Worku et al., 2022) . Unlike the findings in this study, Ahmed and Odisho (2018) found that 100% of the analyzed samples tested positive using RT-PCR. This could be attributed to the study's primers used or local NDV strains.

In reviewing the literature, a few papers addressed the mRNA expression of inflammatory biomarkers or cytokines in NDV-infected chickens. Therefore, RT-qPCR was utilized to observe gene expression changes of several inflammatory biomarkers in NDV-infected birds (Fig. 4). Specifically, the mRNA levels of CRP increased significantly in the liver tissue after two days (48 h) of infection, resulting in about twofold changes. A high CRP level indicated that there is inflammation somewhere in the bodies of birds, and NDV infection seems to be responsible for that rise. Further, CRP has been widely used as an indicator for viral infections such as COVID-19 and it demined disease mortality (Zheng et al., 2020)

The second biomarker measured in this study was interferon gamma (IFN-γ). It is synthesized and secreted by T lymphocytes and natural killer cells and mediates the T-helper type I immune response (Fensterl and Sen, 2009). Furthermore, INF-γ has antiviral roles in birds, including avian flu, ND, and Marek's disease (Swant et al., 2011; Susta et al., 2013). Researchers showed that IFN-γ is able to remove intracellular pathogens, hinder viral replication, activate major histocompatibility complexes I and II, and aid in the processing and presentation of antigens (Yeh et al., 1999; Schultz and Chisari, 1999; Kaiser, 2010). Remarkably, IFN-γ mRNA increased in the infected birds compared with controls, Thus, the possible increase of IFN-γ in the current study is conclusive evidence of the immune response to viral infection with NDV.

The study found that IL-1β mRNA levels increased significantly in the NDV-infected birds compared with controls. IL-1β is a key mediator in the inflammatory reaction during viral infections, resulting in the release of IL-6, which is a molecule that is involved in intercellular and vascular cell adhesion and facilitates lymphocyte activation and leukocytes' infiltration (Peiro et al., 2017). Furthermore, IL-1β can reduce the proliferation of the virus and repair tissue damage, but a high amount of it worsens inflammation and increases lethality rates. The elevation of IL-Iβ can be observed not only in NDV infections but also in other viral infections such as avian flu and infectious bronchitis, as previously documented in other studies (Wang et al., 2016; Thomas et al., 2009; Thi and Hong, 2017; and Amarasinghe et al., 2018). Notably, IL-Iβ reduction was linked to the decreasing severity of pneumonia in H1N1 infection (Kim et al., 2015).

The last biomarker tested in the infected samples was interlukin-6 (IL-6) that is produced in the body during inflammation. The study found high IL-6 mRNA expression in the infected birds compared with the control. IL-6 is an immune protein and pyrogen responsible for fever in infectious and noninfectious diseases. Furthermore, Queiroz et al. (2022) reported that IL-6 is essential for corona virus infection in human, including disease duration and severity. It is synthesized by several types of cells during inflammation, including endothelial cells (Chi et al., 2001), immune cells (Tanaka et al., 2014) and fat cells (Fain, 2010). IL-6 is essential for adaptive immunity development because it helps the differentiation of naïve CD^{4+} T cells (Tanaka et al., 2014). Therefore, the high temperature resulted from

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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).

Conclusion

ND is a devastating viral disease that causes significant economic losses in the research region. The experiment addressed gene expression to observe changes in the mRNA levels of several inflammatory biomarkers. Furthermore, data showed an increase in all the inflammatory biomarker mRNA levels in infected birds compared with controls, and a decrease of the AB titers in the infected birds was found. Moreover, utilizing the molecular approach, PCR, appears to crucial to detect NDV in the samples, and M gene is reliable gene for ND infection.

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Author contributions

Authors contributed equally to perform the Manuscript.

Conflict of interest: We declare no conflict of interest.

Funding: The research received no particular fund.

Data Availability: All data are included within the manuscript.

Table 1. Primer details used in the current experiment.

Target gene	Sequence (5'-3')	Product size	Reference	Accession number
M gene - NDV	F: ATCTATCTGTTCGGGCTCAGTC R: GGCTGTCCCACTGCTAGAGA	107	(Reda & Jasim, 2022)	MZ306221
CRP	F:ATACGTCGCCTTCCACATCC R:CGTTGCCCACCACGTA	149	Provided Kindly from Dr. Sun LAB at University of Arkansas/USA	NM_001313720.3
IL-6	F: TGGTGATAAATCCCGATGAAG R: GGCACTGAAACTCCTGGTCT	191		NM_204628.2
IL-1β	F: GCATCAAGGGCTACAAGCTC R: CAGGCGGTAGAAGATGAAGC	131		XM_046931582.1
IFN-γ	F: AGCTGACGGTGGACCTATTATTGT R: CGGCTTTGCGCTGGATTC	260		NM_205149.2
Gapdh	F: GACGTGCAGCAGGAACACTA R: CTTGGACTTTGCCAGAGAGG	128	(Kadhim et al., 2019)	NM_204305

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