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Expression Profiles of IFN- γ, IL-2 and IL-1β Genes in Village Chickens Naturally Infected with Chicken Anaemia Virus

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ABSTRACT

The cytokines IFN-γ, IL-2 and IL-1β play crucial roles in the immune response against viral infections in chickens. Conventional PCR was conducted using primers that can specifically detect chicken anaemia virus (CAV). The expression levels of these cytokines in 100 village chicken that were naturally infected with CAV were investigated using Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT-qPCR). Tissue samples (thymus, liver, bursa of Fabricius and spleen) were collected from the village chicken in Nigeria and the tissues from each bird were pooled and subsequently subjected to RT-qPCR to determine the expression levels of IFN- γ, IL-2 and IL-1βgenes using glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and 28S as housekeeping genes. The results showed that IFN-ɤ, IL-1β, and IL-2 cytokines had 43.3-, 77.2-, and 85.0- absolute increases, respectively. These findings underscore the interplay of cytokines in orchestrating effective immune responses, offering insights for improving disease management strategies against CAV in chickens.

Keywords: Chicken anaemia virus; Cytokines; Gene expression; Village chicken; Nigeria

INTRODUCTION

Chicken infectious anaemia (CIA) otherwise called anaemia-dermatitis syndrome or blue wing disease (Rozypal *et al.,* 1997) is a viral infection of poultry (mostly 2-4 weeks old chicks) characterized by anaemia, subcutaneous haemorrhage, immunosuppression, cachexia and high mortality (Rozypal *et al*., 1997). It is caused by a chicken infectious anaemia virus (CAV), a single-stranded DNA virus with icosahedral symmetry initially belonging to the family *Circoviridae* (Fenner *et al.,* 1993), however, it is now being classified in the Gyrovirus genus of the family *Anelloviridae* (Li *et al.*, 2017; Rosario *et al.*, 2017; ICTV 2024). Loses in CAV infections are due to poor growth, high mortality and cost of antibiotics used to control the secondary bacterial infection (McNulty, 1991). Susceptibility to the secondary infection is increased in chickens infected with CAV, most probably due to immunosuppressive effect, which leads to impaired development of pathogen-specific T lymphocytes in the host. The principal sites of CAV replication are haemocytoblast in the bone marrow, precursor T cells in the cortex of the thymus and CD8 cells in the spleen (Jordan and Pattison, 1998). Replication in the first stage led to anaemia in the haemocytoblast while replication in the cortex of the thymus and the spleen led to immunosuppression (Aiello and Mays, 1998; Jordan and Pattison, 1998), Viral antigen has also been demonstrated in lymphoid tissues and other organs (Schat, 2003). An experiment in specific-pathogen-free (SPF) chicken, as well as naturally infected chickens with CAV detected the presence of cytotoxic T lymphocytes (CTL) after seven days post infection in chickens with maternal antibody to CAV, however, chickens with no maternal antibody to CAV failed to produce pathogen-specific CTL (Markowski-Grimsrud and Schat, 2003). The cortical lymphocytes are the cells in the thymus which are affected in the beginning of the CAV infection (Jeurissen *et al.,* 1989). However, non-lymphoid leukocytes remain unaffected. Studies suggested that among T-Cell populations, CD8+ cells are more affected than are the CD4+ cells, suggesting that CD8+ cells are more susceptible (Adair *et al.,* 1993). The mechanism of impairment of CTL generation may have resulted from destruction of lymphoid precursor, or indirectly by alteration in essential cytokines in the infected birds (Markowski-Grimsrud and Schat, 2003). It was reported that the virus has minor effects on B-cells and their precursors (Adair, 2000). This indicates that the common lymphoid progenitor cells in bone marrow which provides progenitor cells for seeding of the thymus and Bursa is probably not susceptible to CAV (Adair, 2000). A study observed that CAV infection impairs the generation of cytotoxic T lymphocytes, with associated alterations in cytokine mRNA levels, including interleukin (IL)-1β, IL-

2, and interferon-gamma (IFN-γ) (Markowski-Grimsrud and Schat, 2003). Therefore, this study evaluates the responses of IFN-γ, IL-2, and IL-1β genes in village chickens naturally infected with CAV.

MATERIALS AND METHODS

Sample Collection and CAV Detection

A total of 100 village chickens of varying ages and sexes were conveniently obtained from different parts of Maiduguri, Nigeria. These include Chad Basin (11); University of Maiduguri staff quarters (12); Custom Market (12); Monday market (14); 303 Housing Estate (9); Mairi backyard (12); Dalori Quarters (12); and Gwange Backyard (8); and Malari ward (10). Each chicken was humanly slaughtered in accordance with the International Guiding Principles for Biomedical Research Involving Animals, as established by the Council for International Organizations of Medical Sciences (CIOMS) and the International Council for Laboratory Animal Science (ICLAS) in 2012. The tissue samples (thymus, liver, bursa and spleen) were screened for CAV positive, then pooled positive tissues were stored at -20oC prior to Host mRNA extraction and conventional PCR amplification using specific primers for CAV detection (CUX-standard O3Forward 5' CAAGTAATTTCAAATGAACG-3'; O3Reverse 5'- TTGCCATCTTACAGTCTTAT- 3') as previously reported by Cardona et al. (2000).

Host mRNA Extraction from Tissue Samples

Tissue mRNA was extracted from each homogenized pooled tissue samples collected from the chickens using innuPREP RNA Mini Kit 2.0 (Ajinnuscreen, GmbH, Berlin, Germany) following the manufacturer's protocol. Briefly, homogenized tissue samples were transferred into 1.5 mL reaction tube and incubated for 5 minutes for further lysis under continuous shaking. After lysis, unlysed materials were spun down by centrifugation at maximum speed for 1 minute. The procedure above was repeated twice using a spin filter which was placed into a receiver tube. Approximately 400 µL of 70% ethanol was added to the filtrate from step 2. Each receiver tube with the filtrate were discarded and the spin filter placed into a new receiver tube and finally placed into a new elution tube where the cap of the spin filter was carefully opened and 30-80 µL of RNase free water was added and incubated at room temperature for 1 minute for centrifugation at 11,000g for 1 minute.

Evaluation of mRNA Concentration, RNA Purity and PCR

The purity and concentration of the RNA were confirmed using a UV mini spectrophotometer. The absorbance reading was recorded at 260 nm and 280 nm wavelength for purity of the extracted RNA. The nucleic acid concentration is calculated using the Beer-Lambert law, which predicts a linear change in absorbance with concentration (Hamisu *et al*., 2022).

RT– qPCR Assay for the Quantification of Cytokines

Three genes, namely, IFN-γ, IL-2 and IL-1β were selected in order to estimate their relative mRNA expression from chickens that were naturally infected with CAV. The normalization of IFN-γ, IL-2 and IL-1β genes were carried out using two housekeeping genes: GAPDH and 28S (Jarosinski *et al.*, 2003; Miller *et al.*, 2005). The primer sequences of the target and the housekeeping genes are listed in table 1. Singly Yellow Benzene Ring (SYBR) green qPCR assay was performed using SensiFAST™ SYBR Lo-ROX One-Step Kit (Bioline, Meridian Life Science® Company, U.S.A), and the samples were run on CFX96™ Real-Time System (Bio-Rad, USA) with a final concentration of 20 µL reaction mix. The reaction mixture consists of 10 µL 2x SYBR green One-step mix, 0.8 µL each of 10 µM Forward and Reverse Primers, 0.2 µL reverse transcriptase, 0.4 µL ribosafe RNase inhibitor, 3.8 µL PCR graded deionised distilled water, and 4 µL mRNA template. Three biological replicates were prepared, each of which has three technical replicates of each target gene. The following cycling conditions were used: Reverse Transcriptase at 45 °C for 10 minutes, Polymerase Activation at 95 °C for 2 minutes, Denaturation at 95 °C for 5 seconds, and Annealing/Extension at 60 °C for 20 seconds.

Table 1: Oligonucleotide Primers for SYBR GREEN RT-qPCR used for detection of CAV cytokine genes in village chickens naturally infected with CAV in Maiduguri, Nigeria

Gene		Oligo Sequence	Length	Position
CAV-1	Q5′	5′-GCCCCGGTACGTATAGTGTGAG-3′	22-mer	989–1010
	Q3′	5′-CCGTGAGAAATATGATTCCTTGG-3′	23-mer	1047–1069
IFN-γ	Q5′	5′-AAACAACCTTCCTGATGGCGT-3′	21-mer	408–428
	Q3′	5′-CTGGATTCTCAAGTCGTTCATCG-3′	23-mer	467–489
IL-1β	Q5′	5′-GCTCTACATGTCGTGTGTGATGAG-3′	24-mer	572–595
	Q3′	5′-TGTCGATGTCCCGCATGA-3′	18-mer	634–651
IL-2	Q5′	5′-GATTCATCTCGAGCTCTACACACC-3′	24-mer	191–214
	Q3′	5′-ACCACTTCTCCCAGGTAACACTG-3′	23-mer	249–271
GAPDH	Q5′	5′-TGACGTGCAGCAGGAACACT-3′	20-mer	26–45
	Q3′	5′-GTGACCAGGCGGCCAATAC-3′	19-mer	88–10
28S	Q5′	5′-GGCGAAGCCAGAGGAAACT-3′	19-mer	4703–4721
	Q3′	5′-GACGACCGATTTGCACGTC-3′	19-mer	4746–4764

Data Analysis

The mean quantitative cycle value (Cq) of each target gene was calculated based on the average of the replicates. Then, the target genes were normalized with the reference genes, and expression level of the target genes was calculated using the $2^{-(\Delta\Delta CT)}$ method. First, the C_T values of the target genes were normalized to the C_T of the reference-genes for both the test sample and the calibrator sample, thus:

$\Delta C_{T(test)} = C_{T(target, test)} - C_{T (ref\text{-}gene)}$

$\Delta C_{T(calibrator)} = C_{T(target, calibrator)} - C_{T (ref\text{-}cal);}$

secondly, the ΔC_T of the test samples were normalized to the ΔC_T of the Calibrator, that is,

$\Delta\Delta C_T = \Delta C_{T(test)} - \Delta C_{T (calibrator);}$ and finally, the expression ratio fold increase or decrease was calculated and presented as absolute increase.

$2^{-(\Delta\Delta CT)}$ = Normalized expression ratio (fold difference) (Table 2).

RESULTS

The results showed that out of the total 100 samples,42 (42%) samples were positive for CAV using conventional PCR. Further analysis of the CAV positive pooled tissues indicated that IFN-ɤ, IL-1β, and IL-2 cytokines had absolute increases of 43.3, 77.2, and 85.0 (table 3) respectively. The mean C_T values of the target genes and housekeeping genes in relation to the pooled test tissue samples were presented in table 2.

Table 2: Mean C_T Values of the Target and Housekeeping Genes from Tissue Samples of Chickens Naturally Infected with CAV in Maiduguri, Nigeria

Target gene	Mean C_T Values of			
	Positive (Con)	Housekeeping gene (GAPDH)	Housekeeping gene (28S)	Test Samples
IFN-ɤ	33.8	31.2	29.9	28.33
IL-1β	32.6	33.6	30.9	26.33
IL-2	32.4	32.4	23.9	25.99

Table 3: The absolute increase of the target genes following normalization with the two housekeeping genes from CAV positive tissues collected from chickens in Maiduguri, Nigeria

Target gene	**Absolute increase of target genes**
IFN-ɤ	43.3
IL-1β	77.2
IL-2	85.0

$p \leq 0.05$ significant statistically

DISCUSSION

The result of the present study revealed an absolute increase of IFN-ɤ, IL-1β, and IL-2 cytokines with up-regulated absolute increase of 43.3, 77.2, and 85.0 respectively. IFN-γ is a key cytokine involved in antiviral responses. Its upregulation suggests that the host is mounting an immune response to combat CAV infection. In addition, IFN-γ activates immune cells, such as macrophages and natural killer cells, which are essential for eliminating infected cells and controlling viral replication. Furthermore, it induces the expression of major histocompatibility complex (MHC) class I and II molecules, which are crucial for antigen presentation and the activation of T cells (Hamisu *et al*., 2022). On the other hand, IL-1β is a pro-inflammatory cytokine that plays a key role in initiating the inflammatory response. Its upregulation suggests that the host is responding to CAV infection with an inflammatory response. Finally, IL-2 is essential for T cell proliferation and activation. Its upregulation suggests that the host is mounting a T cell-mediated immune response to CAV infection. The findings in this supports the report of Giotis *et al.* (2018), that showed cytokine gene expression levels in CAV infected chickens of all age groups were found to up-regulate mRNA expression levels in infected chicks 24 hours, 48 hours and 72 hours post infection. This finding is also similar to studies conducted by Giotis *et al.* (2015) that showed an upregulation of IL-Iβ and IL-6 in the thymus and the spleen of CAV infected chicken. IL-2 showed a sustained and substantial increase in mRNA expression levels following infection and only in the thymus and bone marrow of two weeks old chicks. This supports the fact that chicks are most susceptible to disease during their first two weeks of life. Chicken anemia virus is a lymphotropic virus that causes anaemia and immunosuppression in chickens (Giotis *et al.,* 2018). CAV targets erythroid and lymphoid progenitor cells in the bone marrow and thymus respectively, while B-cells appear to be unaffected by the virus (Giotis *et al.,* 2015). Cytokine upregulation during CAV infection plays a critical role in shaping the host immune response. These changes can significantly influence disease progression, immune modulation, and the susceptibility of chickens to secondary infections (Vaziry, 2011). IL-1β and TNF-α are critical in inducing fever and activating macrophages and dendritic cells to enhance antigen presentation. Cytokine imbalance could lead to long-term immunosuppression, impairing the chicken's ability to respond to future infections or vaccines (Adair, 2000). The findings of the current study align with those of Giotis *et al*. (2015, 2018) in several ways: Both studies observed an upregulation of cytokines, including IL-1β, in response to CAV infection. This suggests a consistent immune response to the virus. Furthermore, the studies found that CAV targets lymphoid organs, such as the thymus and spleen, leading to immunosuppression and anaemia. Additionally, Giotis *et al.* (2015) suggest that chicks are most susceptible to CAV infection during their first two weeks of life, which is consistent with the observed sustained and substantial increase in IL-2 mRNA expression levels in the thymus and bone marrow of two-week-old chicks. However, while both studies observed an

upregulation of IL-1β, the current study found a more comprehensive cytokine response, including the upregulation of IFN-γ and IL-2, in contrast Giotis *et al.* (2015) who reported an upregulation of IL-6, but not IFN-γ or IL-2, in the thymus and spleen. Additional contrasting finding between the current study is in relation to cytokine response which the current study found that the cytokine response was not limited to the thymus and spleen, but was also observed in other tissues, such as the liver and bursa. In contrast, Giotis *et al.* (2015) focused primarily on the thymus and spleen. The current study did not specify the age or breed of the chickens used, while Giotis *et al.* (2015) used two-week-old chicks. This difference in age and potentially breed could contribute to the observed differences in cytokine expression patterns. Furthermore, the current study pooled tissue samples from multiple organs, while Giotis *et al.* (2015) analysed individual organs. The sample size and type could influence the results, particularly if the pooled samples masked tissue-specific responses.

Although, there has been several gene expression transcriptomic analyses of other viral infections of chickens, only a few host gene expression studies have been conducted so far following natural infection with CAV. There is no documented study of any kind in the study area, hence, this is the first cytokine gene expression study in village chickens naturally infected with CAV. The findings on cytokine regulation during CAV infection have significant implications for disease control strategies, particularly in the context of village chicken populations in Nigeria. The observed cytokine dysregulation highlights the complex interplay between the virus and the host's immune system, where upregulation of pro-inflammatory cytokines serves as an immediate defense mechanism but may also lead to immunosuppression and increased susceptibility to secondary infections. These dynamics are especially critical in village chickens, which are often exposed to diverse pathogens such as Newcastle disease and infectious bursal disease under resource-limited conditions. Additionally, monitoring cytokine profiles in infected chickens could serve as a diagnostic tool to assess disease progression and the efficacy of vaccination programs. From a broader perspective, these insights emphasize the importance of integrating immunological studies into the management of village poultry. Given that chickens in the study area are majorly kept under free-range system, the risk of CAV transmission is high due to increased contact between infected and susceptible birds. This highlights the need for effective management strategies to control the spread of CAV in Maiduguri. Enhancing awareness and capacity-building among poultry keepers regarding biosecurity and vaccination practices could mitigate the impact of CAV on flock health and productivity.

Conclusion

The study revealed significant upregulation of cytokines IFN-ɤ (43.3-absolute), IL-1β (77.2-absolute), and IL-2 (85.0-absolute) in CAV-infected village chickens, indicating an active immune response. The up regulation was observed in the sampled village chickens irrespective of demographic factors. This pioneering research provides foundational insights into the immunopathogenesis of CAV in village chickens, which may guide future studies on disease control and vaccine development.

Recommendation

There is a need for further studies on gene expression in village chickens within the study area to enhance our understanding, particularly concerning reference controls. Considering the immunosuppressive nature of chicken anemia viruses, whether alone or in combination, it is essential to explore alternative normalization methods beyond housekeeping genes to achieve more accurate assessments.

Conflict of Interests

The authors have no conflict of interest to declare.

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Author's Contributions.

YMS-Idea, research designed, sample collection and literature search. MUS-Data analysis and critical review of manuscript, HIG, MBA and ADE-Supervised the work and provided critical review of manuscript. All authors have read and accepted the final manuscript for publication.

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