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Dynamics of some pro-inflammatory cytokines in commercial cocks experimentally infected with *Pasteurella multocida*

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ABSTRACT

Aim: The study was to determine the dynamics of some pro-inflammatory cytokines in cocks experimentally infected with *Pasteurella multocida*.

Method and materials: A total of 40, eighteen-week-old ISA Brown commercial cocks were used in this study. The birds were randomly assigned to two groups (infected and control) of 20 cocks each. Each bird of the infected group was challenged with dose of 0.5 mL of the inoculum containing $12x10^8$ CFU/mL of *Pasteurella multocida* intra-muscularly using insulin syringes. Blood (3ml) was collected from each of the birds in the infected and control groups via the brachial vein, using 5-mL syringes and 23 G needles, on day 0 (pre-infection), and subsequently, on days 4, 7, 14, 21, 28, 35 and 42 post-infection (pi). Exactly 1 mL of the blood was used for hematological analyses, while the other 2 mL of the blood was used for serum extraction for biochemical assays.

Results: By day seven post infection, all the birds in the infected group showed clinical signs associated with fowl cholera such as ruffled feathers, sneezing, respiratory distress, greenish-yellowish diarrhea, weakness, nasal and ocular discharges.

Conclusion: It was concluded that Significant ($p \leq 0.05$)increase in mean serum concentrations of interferon Gama (IFNγ) and interleukine 1(IL-1β) with significant heterophilia, lymphocytosis and monocytosis signified possible antigenic stimulation of the immune system in response to the experimental infection with *Pasteurella multocida* serotype A:1.

Keywords: Cytokines, Fowl cholera, *Pasteurella multocida*, Leukocytes.

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Introduction

Fowl cholera remains an economically important poultry disease worldwide (Magyar and Lax, 2014; Singh *et al*., 2014; Kardos and Kiss, 2020). The epizootics of the disease (fowl cholera) in the world poses a serious threat to poultry industry (Pandey, 1992; Ambali *et al*., 2003). It causes devastating economic losses to the poultry industry through weight loss, condemnations or downgrading of carcasses, reduction of fertility and death world-wide (Aye *et al*., 2001; Lee *et al*., 2012; Barkhordari *et al*., 2013, Joseph *et al*., 2019).

The mode of transmission of the disease can be by mechanical means through vectors, aerosol and ingestion of contaminated feed and water. Most farm animals may be carriers of *Pasteurella multocida* (Blackall, 2003; Susan and Micheal, 2016; Abdu and Musa, 2019). The disease, fowl cholera is a septicemic disease that is responsible for the widespread organs and tissues damage in infected birds. Three clinical forms of the disease in poultry have been identified; namely, per-acute, acute and chronic forms. The per-acute form is associated with the most virulent and highly infectious organism; birds in good conditions are suddenly found dead with no premonitory signs. In the acute form, chickens will show anorexia, mucus discharge from the beak, high fever, loss of weight,

drop in egg production, cyanosis of wattles and comb, green fetid diarrhea (Rosales, 2013; Joseph *et al*., 2019; Jarkko, 2021). The chronic form of the disease is associated with conjunctivitis, swollen wattles, tracheitis, lameness, and dyspnea, swelling of joints and tendon sheaths of legs and wings, torticollis, oophoritis with regression of ovarian follicles, drop in egg production and hatchability problem in female birds, orchitis in the male birds and mortality rates of 5-25 % which could rise to 45 % (Susan and Michael, 2016; Joseph *et al*., 2018; Abdu and Musa 2019, Jarkko, 2021). Gross lesions in infected chickens with *P. multocida* include petechial and ecchymotic hemorrhages on coronary fats of the heart, proventriculus, gizzard, peritoneum, intestines, and abdominal fats. The liver is frequently enlarged, congested and streaked with multiple pinpoint greyish necrotic foci and there is splenomegaly and congestion of ovarian follicles (Yakubu *et al*., 2015; Abdu and Musa, 2019; Joseph *et al*., 2019). Histopathological changes of fowl cholera include, haemorrhage, congestion and moderate to severe lymphocytic, heterophilic and macrophagic cellular infiltration in the liver, heart and lungs (Shilpa *et al*., 2006; Dashe *et al*., 2015; Yakubu *et al*., 2015, Joseph *et al*., 2018).

Cytokines are proteins or peptides secreted by cells which play a key role by stimulating immune and inflammatory responses through the activation and regulation of other cells and tissues. Cytokines are generally classified according to their activity and/or the cell they are produced by or act upon (Siatskas *et al*., 2000; Jurisic, 2020). Most often, they can be classified into: pro-inflammatory cytokines, interferons, and colony stimulating factors (Kogut, 2000; Hilton *et al*., 2002; Jurisic, 2020). Pro-inflammatory cytokines such as IFNγ, IL-1β, IL-6, IL-8 and IL-17 are credited with a role in inflammatory responses (Kaiser and Stabeli, 2008). Pro-inflammatory cytokines are cytokines that are produced by both phagocytic and non-immune cells at the site of inflammation or acute phase response due to infection, disease or tissue trauma (Al-Khalaifah and Al-Nasser, 2018). However, they are produced predominantly by activated macrophages and are involved in the upregulation of inflammatory reactions (Jun-Ming Zhang and Jianxiong, 2009). The commonly described poultry pro-inflammatory cytokines are interleukin Ibeta (IL-1β), interleukin 2 (IL-2), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-18 (IL-18), tumour necrosis factor beta (TNF-β) and interferon Gama (IFNγ) (Michael, 2000). Their production is stimulated by macrophages, following activation by bacterial products.

Materials and Methods

The study was carried out in the Department of Veterinary Pathology, Faculty of Veterinary Medicine, Ahmadu Bello University Samaru, Zaria, which is located in SabonGari Local Government Area of Kaduna State. Samaru is located within the Northern Guinea Savannah zone of North western Nigeria (Hamma *et al*., 2014). It lies between latitude 7^0 and 11^0 N, and longitude 7^0 and 44^0E and has an average rainfall between 1,000 to 1,250 mm and an average temperature between 17 ^{0}C to 33 ^{0}C and a vegetation cover of predominantly trees and grasses (Hamma *et al*, 2014).

Ethical Approval

Ethical approval for the use of animals was obtained from the Ahmadu Bello University (ABU) Committee on Animal Use and Care (ABUCAUC) with the approval number ABU/2023/156.

Experimental Birds

A total of 40 eighteen-week-old ISA Brown cockerels, were acquired from a farm in Jos. The cockerels were brooded for 5 weeks and reared for 18 weeks at Joemed integrated poultry farm, Zawan, Jos, Plateau State. The cockerels during brooding were immunized against all vaccinable endemic diseases other than fowl cholera after which they were transported by road to Ahmadu Bello University, Zaria. The birds were kept intensively in deep liter for 5 weeks to acclimatize to the new environment, get to sexual maturity (23 weeks) in washed and disinfected poultry research pens of veterinary teaching hospital Ahmadu Bello University, Zaria. During the acclimatization period and throughout the experimental period, the birds were fed standard commercial growers mash (Hybrid feeds®) and drinking water provided ad libitum.

Source of bacterial organism

The challenge bacterium, *Pasteurella multocida* serotype A: 1, (vaccine strain) used in this study was provided by the Department of Bacteriology, National Veterinary Research Institute, Vom, Plateau State, Nigeria.

Sub-culture of the organism

The bacterium from the previously prepared slant

was reactivated once again in the laboratory by sub-culturing on blood agar. The colonies which resulted were then examined for the characteristic features, color and morphology and tested for gram stain reaction (Gram negative). McFarland turbidity standards as reviewed by Acharya (2016) were made in the laboratory by preparing a 1% solution of sulfuric acid and they were mixed in order to obtain a barium pricipate. The volumes of the two reagents were adjusted to prepare standards of different turbidities that represent different concentrations of bacterium. The standards were used to visually compare the turbidity of a suspension of bacterium.

Pre-infection bacteriological monitoring of experimental birds

During the acclimatization period, just before commencement of the experiment, oropharyngeal swabs were taken from all birds in both groups and used to verify that they were free of *P. multocida* infection. The oro-pharyngeal swabs were collected from the experimental birds in both groups which were then used to inoculate an already prepared blood agar after which they were incubated at 37 ^{0}C for 24 hours. The resulting colonies (growth) on the blood agar were subjected to indole and sugar tests according to standard laboratory methods (Glisson *et al*., 2008; Dashe *et al*., 2015)

Challenging of the birds with Pasteurella multocida

After reaching sexual maturity at 23 weeks old, the birds were assigned randomly into two groups (infected and uninfected) of 20 cocks each at 25 weeks old. On the day of infection (Day 0) each of the birds in the infected group was challenged by injecting a dose of 0.5 mL of the inoculum containing 12x10^8 CFU/mL of *Pasteurella multocida* intra-muscularly using sterile insulin syringes and needles (Amany and Abda-Alla, 1997; Dashe *et al*., 2015; Joseph *et al*., 2019).

Clinical Observation

Following inoculation of the birds from day 0 post infection (pi), and throughout the experiment that lasted for six weeks, the experimental birds were monitored closely for clinical signs of fowl cholera on daily basis and all findings were recorded appropriately.

Collection of blood for hematological evaluations

Blood (3mL) was collected from each of the birds in the infected and control groups via the brachial vein, using 5-mL sterile syringes and 23 G needles, on day 0 (pre-infection), and subsequently, on days 4, 7, 14, 21, 28, 35 and 42 post-infection (pi). The blood collection was carried out in the morning (08.00 to 09.00 hours). Each time, 1 mL out of the 3 mL of blood collected was dispensed into ethylenediaminetetraacetic acid (EDTA) coated sample bottle and was used for haematological analyses in the Clinical Pathology Laboratory in the Department of Veterinary Pathology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Kaduna State, Nigeria.

Determination of biochemical parameters

Blood samples of 2 mL out of the 3 mL collected from each bird in the infected and control groups was put into a plain sample bottle, with no anticoagulant for serum extraction. It was centrifuged for 10 minutes at approximately 1000 xg to obtain serum, which was transferred into a labelled tube and then stored at -20^{0}C for biochemical assays of interleukin 1 beta (IL-1β) and interferon (IFN-γ) using chicken specific enzyme linked immunosorbent assay (ELISA) kits for IL-1β and(IFN-γ) (ELK {Wuhan} Biotechnology Co, Ltd).All reagents were prepared prior to commencement of the assay procedure and adjusted to room temperature. After following the protocol, the optical density (OD) read at 450 nm using a microplate reader Rator (RT-2100C),that was carried out atKayomeg Medical Diagnostic Centre, No 4 Ribadu Road off Kofar Gamji adjacent to Al-Manar Mosque Kaduna, Kaduna State, Nigeria.

Bacteriological isolation

At necropsy, swabs of blood were aseptically taken from the hearts, while tissues from liver, kidneys and spleen were also collected from the *P. multocida* infected cocks on days 4, 7, 14, 21, and 28 pi for bacterial isolation according to a standard laboratory procedure (Dashe *et al*., 2015;WOAH, 2015).

Statistical Analysis

All data obtained were subjected to statistical analysis including the calculation of the means and standard error of the means. Data between groups were evaluated by Student t-test and values of $P < 0.05$ were considered significant using Graph Pad Prism Version 8.00 for Windows, Graph Pad Software, San Diego California, USA.

Results and Discussion

Clinical manifestation of fowl cholera in infected commercial cocks

Following infection with *Pasteurella multocida* serotype A:1, birds in the infected group appeared

clinically normal until day 7 pi when the birds started passing out greenish diarrhea with drop in feed and water consumption, other clinical signs of fowl cholera, such as ruffled feathers, siting on hock, weakness, paleness of wattles and combs, loss of weight, oral and nasal discharges and dyspnea in the infected group. There was mortality rate of 25% in the infected group while the birds in the control group showed no clinical signs of the disease throughout the experimental periods.

Bacterial recovery from the infected birds

Pasteurella multocida was isolated from the heart, liver, and spleen of the infected birds as from day 7 pi and throughout the experimental period. The *P. multocida* was confirmed after subjecting the sub-colonies on blood agar according to standard biochemical test as described for *P. multocida* (Dashe *et al*., 2015; Joseph *et al*., 2019).

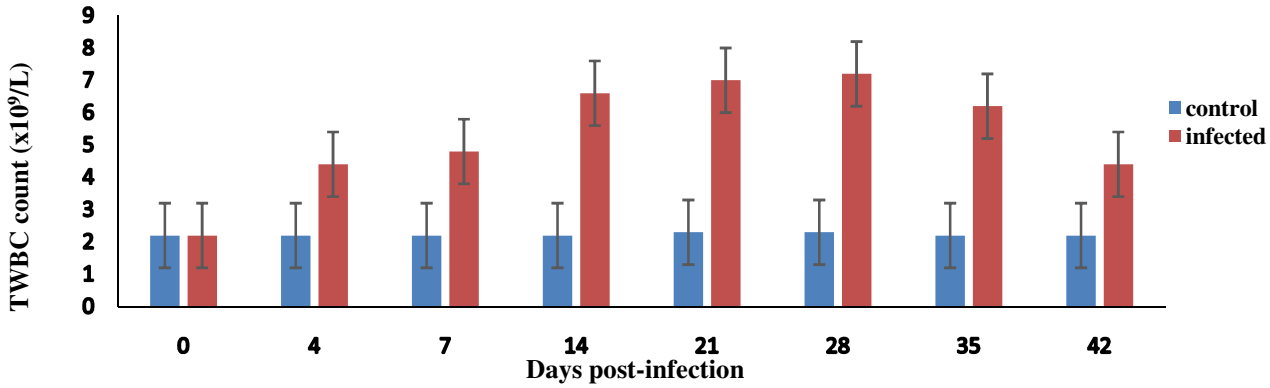

Fig 1. Mean (± SEM) total white blood cell counts of cocks experimentally infected with *P. Multocida* and control cocks.

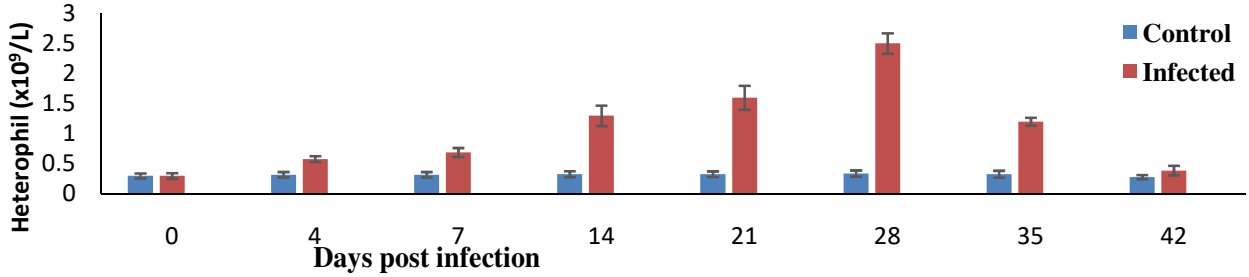

Fig 2. Mean (± SEM) heterophil counts of cocks experimentally infected with *P. Multocida* and control cocks.

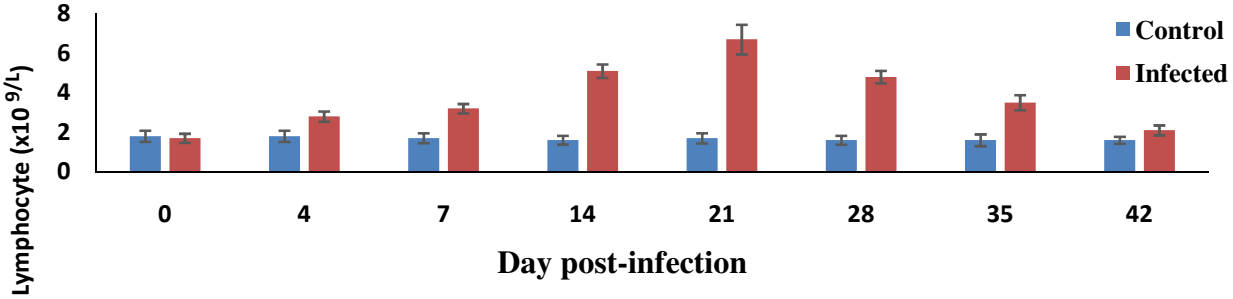

Fig 3. Mean (± SEM) lymphocytes counts of cocks experimentally infected with *P. multocida* and control cocks.

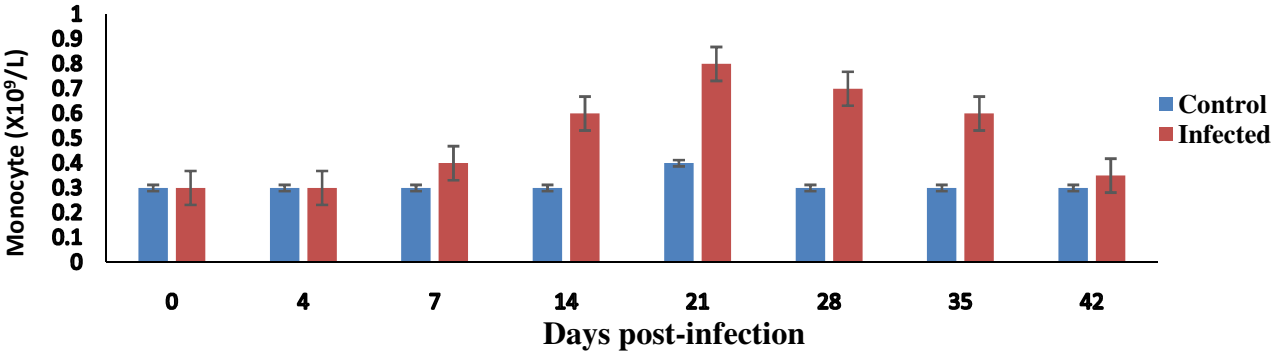

Fig 4. Mean (± SEM) monocytes counts of cocks experimentally infected with *P. Multocida* and control cocks.

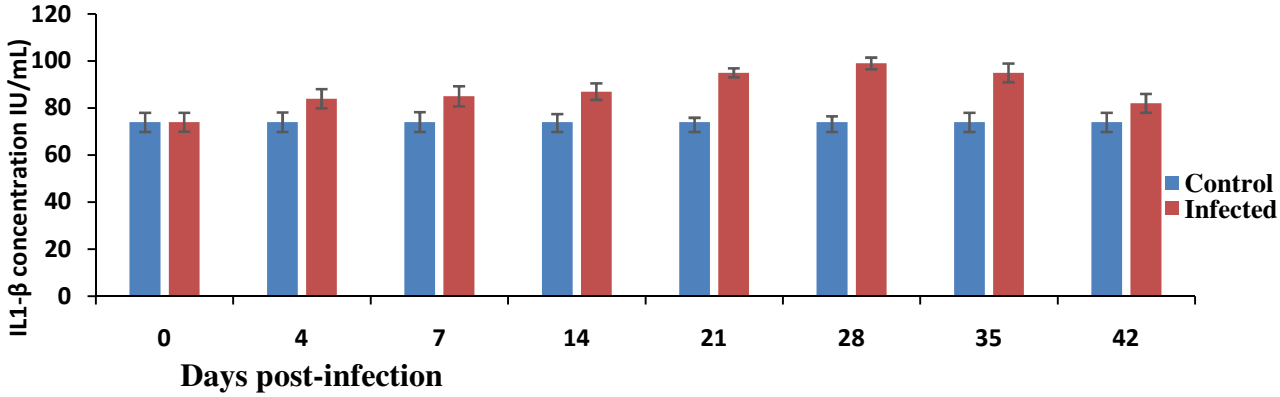

Fig 5. Mean (± SEM) interleukin 1 beta concentrations of cocks experimentally infected with *P. Multocida* and the control cocks.

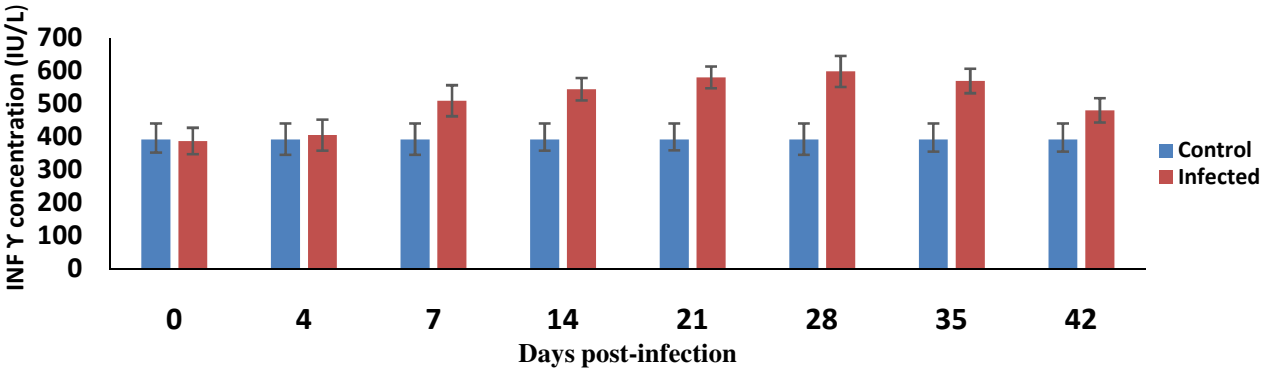

Fig 6: Mean (± SEM) interferon gama concentrations of cocks experimentally infected with P. Multocida and control cocks.

The clinical signs observed in the *Pasteurella multocida* experimentally infected cocks in this study, which included greenish-yellowish diarrhoea, dehydration, anorexia, ruffled feathers, dyspnoea with nasal discharge, respiratory distress, weakness, paleness and cyanosis of wattles and combs were in consonance with several previous literature on fowl cholera (Christensen, 2013; Dashe *et al*., 2015; Abdu, 2014; Joseph *et al*., 2018; Jarkko, 2021). Of 25% mortality rate recorded in this study corresponded with the range of 5-25 % which could rise to 45 % reported previously (Gustafson *et al*., 1998; Susan and Michael, 2016; Abdu and Musa 2019, Jarkko, 2021). The leukogram of the *P. multocida*-infected cocks showed significant leukocytosis that was characterised by heterophilia, lymphocytosis, and monocytosis in the early period of the infection. The heterophils form the first line of innate cellular defence against pathogens and they actively participate in inflammation, whereby

they detect and destroy pathogenic microbes using a repertoire of killing mechanisms such as phagocytosis, cellular degranulation or oxidative burst. The heterophilia that was significantly noticed as from days 4 to 42 pi in the *P. multocida* infected cocks in this study possibly signifies that the chickens were responding to the inciting agent due to antigenic stimulation caused by the *P. multocida* (Panna *et al.*, 2015; Dashe *et al*., 2015; Joseph *et al*., 2018). The antigenic stimulation could be responsible for the early release of inflammatory cells, especially heterophils into the body system in order to phagocytise and kill the offending organism as reported by Vegad and Katyar (1995). Similarly, a progressive and significant decrease in the mean heterophils count on days 35 and 42 pi could be as a result of shift of cells (heterophils) from the circulating pool into the marginal pool and then into the body tissues by diapedesis as reported by Howes *et al.* (1978); Vegad and Katyar, (1995); Joseph *et al*. (2018) in order to phagocytise the offending organism. The significant increase in the mean lymphocytes count that was observed on days 4 to 42 pi indicates lymphocytosis which means that *P. multocida* stimulates lymphocytosis, which was similarly reportedby Mitchell and Johns, (2008); Joseph *et al*. (2018). Another possible reason for lymphocytosis in this study could be that the birds were recovering from infection with *P. multocida* as lymphocytosis had been reported in the recovery stage of most infectious diseases as reported by Campbell, (1994). The monocytosis that was detected on days 7 to 42 pi buttressed the fact that there was a possible increase in the demand for macrophages in order to mop up tissue debris that emanated from inflammatory reaction in the *P. multocida* infected cocks which corresponded with the reportby Mckinney and Rebers (1980), in *Pasteurella multocida* non-immunized turkeys and fowls. The mean serum interleukin 1 beta (IL-1β) concentration in the infected chicken progressively showed significant ($p \leq 0.05$) increase from day 4 pi up to day 35 pi, this could be due to the fact that the biological concentration of interleukin 1 beta is highly inflammatory in a disease condition since its main function is to activate the immune system in an acute phase response. However, IL-1β activates a range of cells including macrophages and lymphocytes that may thus lead to production of other cytokines and chemokines such as IL-8 and so on. Therefore, the increase in serum concentration of, IL-1β in this present study, possibly resulted from antigenic stimulation following infection with *P. multocida* which afterwards led to the release of macrophages and lymphocytes due to activation by bacterial products. These findings were similarly reported by Lloyd and Oppenhein. (1992), Xing *et al*. (1994) and Micheal (2000). The mean serum interferon Gama concentration (IFN-γ) in the infected chicken progressively and significantly($p \leq 0.05$) increased from days 4 to 35 pi in the infected group may be due to antigenic stimulation resulting from the infection which thereafter, led to activation of macrophages and lymphocytes to produce more cytokines. However, in this present study, for the fact that IFN-γ and IL-1β activities were significantly ($p \leq 0.05$) higher when mean serum concentrations for heterophils, lymphocytes and monocytes were also significantly higher, buttressed the fact that both pro-inflammatory cytokines (IFN-γ and IL-1β) possibly activated macrophages to produce other cytokines which might have led to an improved immune response to the disease condition which was similarly reported by De Maeyer and De Maeyer-Guignard (1998). Therefore, the combined effects of significant ($p \leq 0.05$)increase in serum concentrations of IL-1β and IFN-γ, especially on the day 28 which they got to their peak levels could be that both pro-inflammatory cytokines(IL-1β and IFN-γ) might have contributed partly in strengthening of the immune system in the infected cocks which was similarly reported by Lambretcht (1999), and no death was recorded from day 28 to day 42 pi when the research was terminated. These two pro-inflammatory cytokines (IL-1β and INF-γ) are known to be active pro-inflammatory cytokines that are produced immediately following infection or tissue injuries and which thereafter, stimulate an interactive network of biological responses, including effects on the other cells and on cytokines themselves.

Conclusion

It was concluded that significant ($p \leq 0.05$) increase in mean serum concentrations of (IFNγ) and IL-1β with significant heterophilia, lymphocytosis and monocytosis signified possible antigenic stimulation of the immune system in response to the infection with *Pasteurella multocida.*

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