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Full Length Research

Immunohistochemical evaluation of the expressed immunoglobulin M (IgM)-positive lymphocyte during the development of thymus in Nigerian native chicken

Nnadozie, O.[1]*, Nwaogu, I. C.[2], Igwebuike, U. M.[2] and Ikpegbu, E.[1]

[1]Department of Veterinary Anatomy, Michael Okpara University of Agriculture, Umudike. Abia State, Nigeria.
[2]Department of Veterinary Anatomy, University of Nigeria, Nsukka. Enugu State, Nigeria.

*Corresponding author. Email: drdozie15@gmail.com; Tel: +2347088871604.

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ABSTRACT: Immunoglobulins (Igs) or antibodies are glycoproteins synthesised by activated B lymphocytes that combat antigens. Among the three isotypes in chicken, immunoglobulin M (IgM) is the first expressed antibody following exposure to a novel antigen. The thymic expressions of IgM-positive lymphocytes were studied in Nigerian native chicken using immunohistochemistry to determine the relative time of appearance of IgM-positive lymphocytes without antigenic stimulation, as well as its periodic magnitude of occurrence in both embryos and unvaccinated chicks. Fifteen chicken embryos and twenty-five chicks were used for the study. Hatchable eggs laid by apparently healthy free-range hens were incubated in an electric egg incubator, and the embryos were harvested. The hatched chicks were raised in a deep-litter pen. The thymus of both embryos and post-hatch chicks was collected through a ventral neck incision at embryonic incubation day 10 (EID 10), EID 14, EID 18 and at hatch or day 0 (D 0), D 7, D 14, D 28, D 42 and fixed in neutral-buffered formalin. The fixed tissues were processed for immunohistochemical study using the indirect immunoperoxidase technique. IgM-positive lymphocytes appeared in the thymus at about EID 10. Throughout the embryonic periods up to the point of hatch, there were mild expressions of IgM-positive lymphocytes. Relatively moderate expressions were observed between D 7 and D 14 post-hatch, while there were marked expressions at D 28 and D 42. IgM-synthesising lymphocytes appeared early in the embryonic thymus, and its occurrence in substantial quantities in the post-hatch chicks' thymus indicates strong humoral immunological tendencies in growing Nigerian native chicken.

Keywords: Nigerian native chicken, thymus, Immunoglobulin-M, immunohistochemistry, lymphocyte, embryo, unvaccinated chicks.

INTRODUCTION

The avian immune system is composed of several lines of defence to prevent pathogen entry and subsequent infection (Erf, 2003, 2007). In active immunity, certain proteins called antigens or their modifications will preferentially lead to the development of either cell-mediated immune responses or humoral immunity against the antigen. The endogenous antigens or intracellular pathogens often elicit cell-mediated immune responses that work to destroy the infected cell or enter the cell to eliminate the antigen (Erf, 2003). The specialised cells known as T lymphocytes produced in the thymus are the primary cells active in cell-mediated immunity (Qureshi *et al*., 1998; Davison *et al*., 2008). The humoral immune response involves the secretion of specific proteins known as antibodies by the plasma cells, the transformed type of B lymphocytes produced in the bursa of Fabricius. These antibodies, usually found in body fluids and tissue spaces, are most effective in eliminating extracellular pathogens (Lillehoj and Trout, 1996; Hussan *et al*., 2009; Bonilla and Oettgen, 2010).

The humoral immune responses are initiated following the recognition of an antigen by antigen-specific B lymphocytes. To activate B cells, the antigen binds with specific protein receptors on the surface of the cells. The

B cell activation leads to the proliferation of antigen-specific cells and their differentiation into memory and antibody-secreting cells, which subsequently synthesise the immunoglobulins that are capable of identifying and neutralising pathogens such as bacteria and viruses (Litman *et al*., 1993; Janeway, 2001; Abbas *et al*., 2014). However, natural immunoglobulin-positive lymphocytes without antigenic stimulation occur in various lymphoid tissues of developing chick embryos and post-hatch chicks (Islam *et al*., 2013), signifying the innate potential of antibody synthesis in chicken.

The immunoglobulins or antibodies are glycoproteins with three different isotypes, namely: Immunoglobulin-A (IgA), Immunoglobulin-M (IgM) and Immunoglobulin-Y (IgY) in chickens (Litman *et al*., 1993). Specifically, Immunoglobulin M has long been recognised as the most predominant B-cell antigen receptor and the first avian antibody isotype expressed during embryonic development (Davison *et al*., 2008; Islam *et al*., 2013). It was also reported to be the major antibody produced following an initial contact with a novel antigen, being more active than the other isotypes in opsonisation, agglutination, virus neutralisation and complement activation (Ritchie *et al*., 1994).

The indigenous breeds of chicken, such as the Nigerian native chicken, have been associated with innate potentials of disease resistance (Sonaiya *et al*., 1999; Singh *et al*., 2004; Ekei *et al*., 2019). Considering the significant role of Immunoglobulin-M in the initiation of humoral immune responses in chickens, its involvement in the overall immunocompetence expressed by the native chickens cannot be overemphasised.

Although in the avian thymus a greater percentage of the primary immunological cells are T-lymphocytes, which are not endowed with antibody production potentials, a subpopulation of B cells that can differentiate into Ig-containing plasma cells for local defensive functions exists in the thymus, just as a T-lymphocyte subpopulation in the bursa of Fabricius (Masum *et al*., 2014). Generally, information on the timeline of emergence of IgM-positive lymphocytes and its periodic magnitude of occurrence in the developing thymus of Nigerian native chicken is limited. Thus, this study aims to determine the approximate time of appearance of IgM-positive lymphocytes and its relative amounts at different stages of development of the thymus.

MATERIALS AND METHODS

A total of fifteen chick embryos and twenty-five post-hatch chicks of either sex were used for the study. However, one hundred and twenty hatchable indigenous chicken eggs were acquired from apparently healthy laying hens raised by free free-range backyard method in Ovim community, Isuikwuato Local Government Area of Abia State, Nigeria. The eggs were incubated in an electric egg incubator at 37°C and 55 – 60% relative humidity (Yoshimura *et al*., 2009; Oznurlu *et al*., 2010).

Five chick embryos were harvested from the gravid eggs on each of Embryonic Incubation Day (EID) 10, EID 14 and EID 18 by cracking the gravid egg shell around the vertical midline with a knife edge, and the transverse diameter was cut using a surgical scissors to expose the embryo. The yolk sac was detached from the embryo by severing the stalk, and foetal debris was removed. The rest of the eggs were incubated until hatching for the post-hatch studies.

Following hatching, the chicks were housed in a deep-litter pen in the poultry unit of the College of Veterinary Medicine, Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria. The chicks were fed commercially compounded feed (Topfeed^{R}, broiler chick mesh), and water was given *ad libitum*. No medications, including vaccinations, were given throughout the study period.

Five randomly selected chicks were sacrificed at hatch or day 0 (D 0) and on each of D 7, D 14, D 28 and D 42 post-hatch by cervical subluxation. The thymus of both embryos and post-hatch chicks was collected through a ventral neck incision using the approach of Alboghobeish and Mayahi (2003), and fixed in neutral buffered formalin.

Immunohistochemical investigation

The immunohistochemical technique involves two different procedures: histological sample preparation and the indirect immunoperoxidase processing of the histological samples.

Histological sample preparation

The fixed specimens were processed by placing them in ascending grades of ethanol in the following order: 70% for 1 hour, first 95% ethanol for 1 hour and second 95% ethanol for 1¼ hours, first absolute ethanol for 1½ hours, second absolute ethanol for 2 hours and third absolute ethanol for 2 hours to ensure proper dehydration of the tissues. It was then transferred to a mixture of equal volumes of ethanol and xylene, where it was left overnight. It was later cleared in two changes of xylene for 1 hour each. It was then infiltrated for 1 hour with molten paraffin wax (salsa wax) in the oven at 60°C. The tissues were embedded in paraffin wax, trimmed and mounted on a wooden chuck, and then sectioned at 5 μm thickness using a microtome (Leitz Rotary microtome, 15120). The sections were floated in a floating-out bath from where they were picked with clean, charged slides.

Indirect Immunoperoxidase processing

The histological samples were deparaffinized in a heating cupboard at 60°C for 30-45 minutes and rinsed in 2 changes of xylene for 15 minutes each. Sections were then hydrated in descending grades of alcohol in the following order (99%, 96% and 70%) for 3 minutes each and rinsed shortly in distilled water. Sections were transferred to a solution of Tris Buffer Saline (TBS) and hydrogen peroxide (1 ml 30% H_2O_2/ 9 ml TBS) for 15 minutes to block endogenous peroxidase. Sections were heated at 99°C for 15 minutes in citrate buffer solution for antigen or target retrieval, and allowed enough time to cool to room temperature. Sections were then rinsed in three changes of a solution comprising 0.2% skim milk powder (CAS-NO: 999999-99-4. Merck KGaA, 64271) and 0.3% Triton-X in TBS (Buffer 1) for 15 minutes per change by placing a few drops of Buffer 1 solution on the sections in the immunohistochemical chamber to block non-specific binding of the antibody. The sections were overlaid with a mixture of 0.3% Triton-X in TBS and goat anti-chicken immunoglobulin M (AB.com, N0-Ref 8282908, Bat Lot 35527; 1:550) and incubated in a moisturising chamber for 18 hours at 4°C. Samples were brought out after 18 hours and rinsed in 0.3% Triton-X in TBS (Buffer 2) to remove excess primary antibody. After rinsing, sections were treated with rabbit anti-goat immunoglobulin M (1:400) and left for 70 minutes at room temperature. Sections were then rinsed in three changes of 50mM TBS buffer, pH 7.4 (Buffer 3) for 10 minutes each, followed by immersion in 0.2mg 3,3'- diamino-benzidine-tetrahydrochloride dehydrate (DAB) per ml of Tris-hydrochloride (0.05M, PH 7.6) containing 0.03% hydrogen peroxide to reveal positive reaction for IgM. The DAB was washed off the sections in three changes of TBS for 10 minutes each, and the sections were counterstained with Mayer's haematoxylin for 2 minutes. Sections were finally rinsed in running tap water and dehydrated in ascending grades of ethanol in the following order (70%, 90% and 99%), followed by immersion in two changes of xylene for 10 minutes each, and coverslips mounted. Slides were studied under a light microscope (BestSco, e; China), and selected images were captured using a Moticam 2.0 digital camera attached to a computer. The magnitude of expression of IgM-positive lymphocytes was evaluated as mild (+), moderate (++), and marked (+++).

Ethical clearance

Ethical approval was obtained from the Research Ethics Committee of the College of Veterinary Medicine, Michael Okpara University of Agriculture, Umudike. The Ethical Approval Reference Number is MOUAU/CVM/REC/202526.

RESULTS

During the embryonic periods, the thymus expressed mild IgM-positive lymphocytes that were sparsely distributed in single cell units as well as in clusters across the parenchyma at EID 10 (Figure 1), while at EID 18, the few activated cells were principally located in the thymic medulla (Figure 2).

At hatch (D 0), the expression of IgM-positive lymphocytes remained mild. The IgM-positive cells were diffusely distributed in the parenchyma, with a greater number located in the thymic medulla (Figure 3).

By D 7, the IgM-positive lymphocytes expression remained mild. The few activated cells were randomly distributed in approximately equal proportions within the thymic cortex and medulla (Figure 4).

At D 14, there was moderate expression of IgM-positive lymphocytes. Large numbers of IgM-positive lymphocytes were found accumulated in the subcapsular regions of the parenchyma (Figure 5).

At D 28, there was marked expression of IgM-positive lymphocytes. The activated cells were in clusters of variable densities across the tissues of the parenchyma (Figure 6).

By D 42, the IgM-positive lymphocyte expression remained marked. The IgM-positive cells still appeared in clusters in the cortex and medulla of the parenchyma, and most of the positive cortical cells dominated the subcapsular regions (Figure 7).

DISCUSSION

Experimental evaluations showed that exposure of chickens to infectious organisms or antigenic substances stimulates accelerated production of antibodies (Ekei *et al.*, 2019). However, chickens naturally possess the ability to produce antibodies without antigenic stimulation. Therefore, it is possible to assess the immunological competence of a chicken through evaluation of self-enhanced periodic levels of immunoglobulin-positive lymphocytes in the lymphoid organs. This forms the basis of this research.

It is worthy of note that IgM is the most predominant B-cell antigen receptor and the first avian antibody isotype expressed during embryonic development (Davison *et al.*, 2008; Islam *et al.*, 2013). Also, it is the major antibody produced following an initial contact with a novel antigen (Ritchie *et al.*, 1994). Therefore, its periodic concentration in developing lymphoid organs can demonstrate the innate pattern of development of the humoral immune mechanism in Nigerian native chicken.

Although in the thymus a greater percentage of the primary immunocompetent cells are T-lymphocytes not endowed with antibody production potentials, a percentage of the thymic cell population under natural conditions is B-lymphocytes that have the potential of becoming activated into Ig-containing plasma cells (Khan and Hashimoto, 1996; Masum *et al.*, 2014).

The result of this study showed that IgM-positive

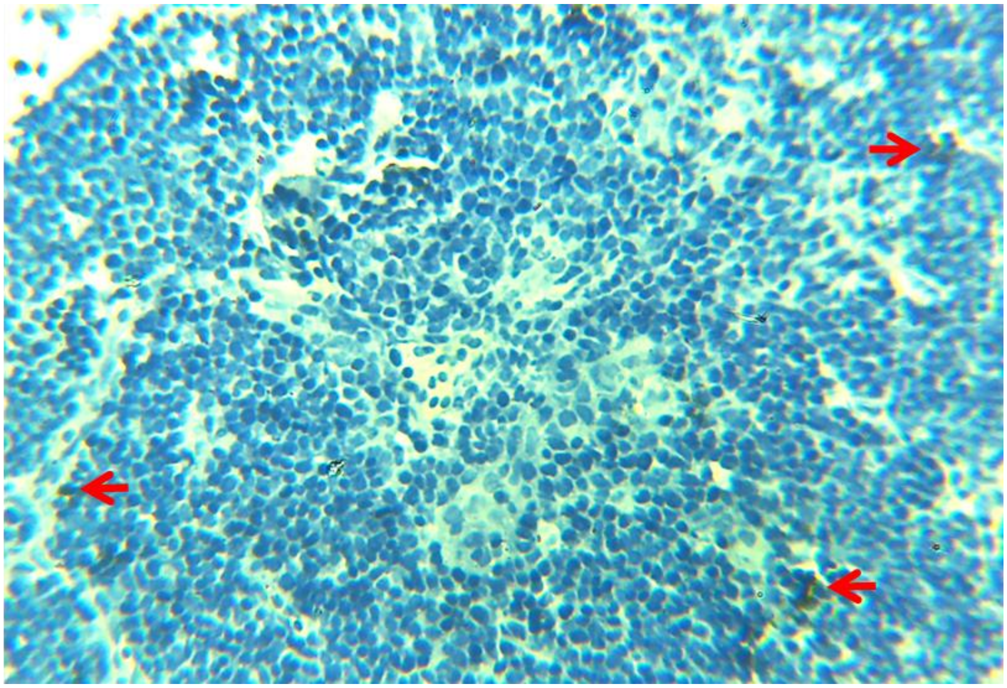

Figure 1. Photomicrographs of immunostained section of the thymus at EID 10 showing the parenchyma. Note the IgM-positve lymphocytes (arrow) within the thymic cell population; X400.

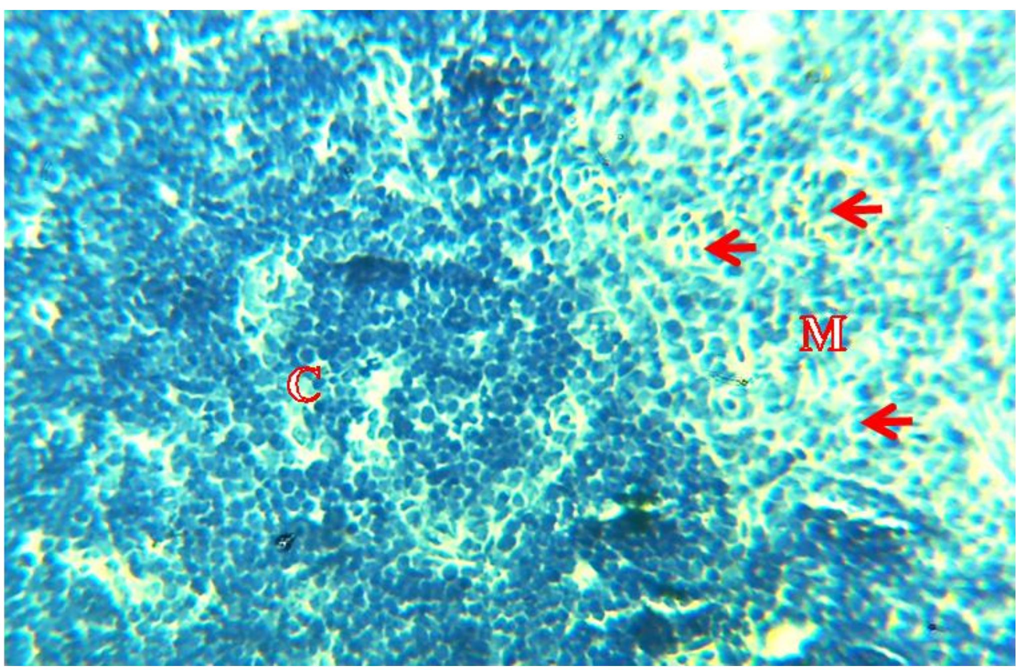

Figure 2. Photomicrographs of immunostained section of the thymus at EID 18 showing the parenchyma. Note the IgM-positve lymphocytes (arrow) in the thymic medulla; M. C: cortex; X400.

lymphocytes appeared in the thymus as early as EID 10. Though in low magnitude, the early appearance of these IgM-positive cells in the thymus where B lymphocytes are of low concentration indicates high humoral immune potentials in Nigerian native chicken. Islam *et al*. (2013) observed similar wave of IgM-positive lymphocytes in the thymus of native chicken of Bangladesh, but at a later embryonic age of EID 20. It was revealed that B

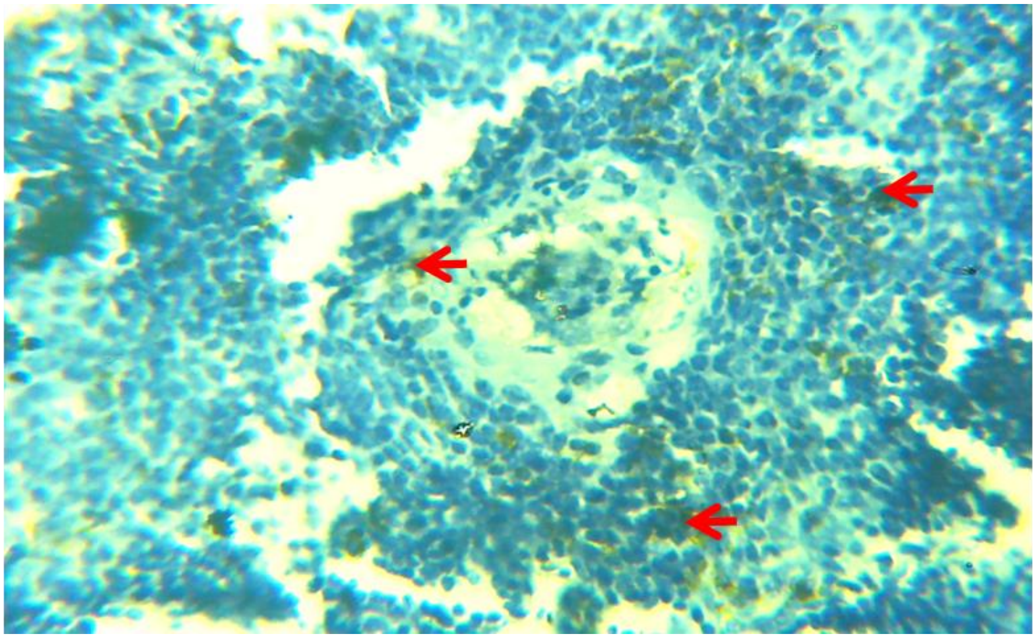

Figure 3. Photomicrographs of immunostained section of the thymus at hatch showing the thymic parenchyma. Note the IgM-positve lymphocytes (arrow) within the thymic cell population; X400.

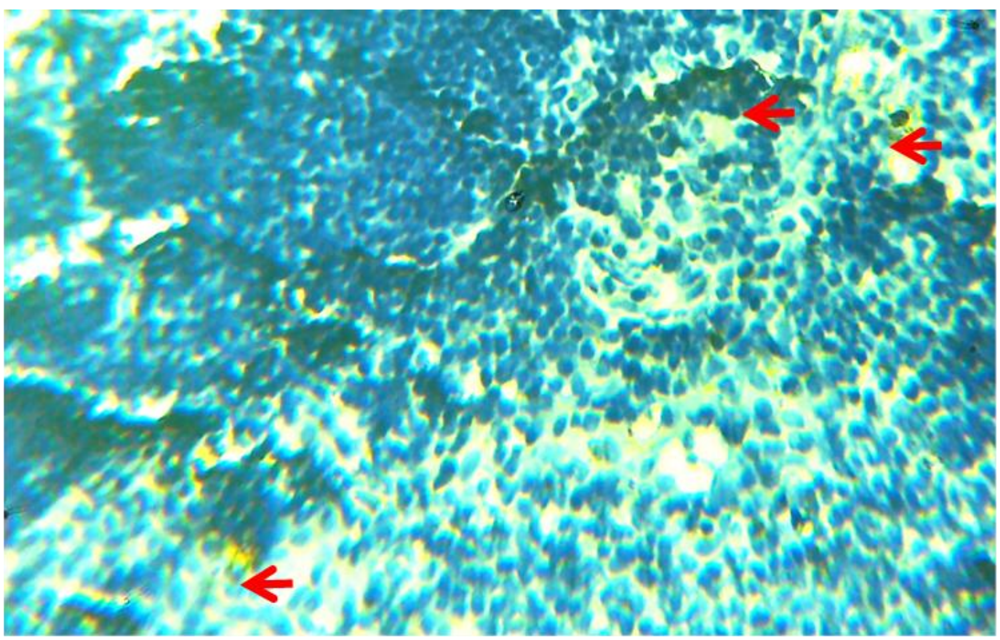

Figure 4. Photomicrographs of immunostained section of the thymus at D 7 post-hatch. Note the IgM-positive cells (arrow) within the parenchyma; X400.

lymphocytes first synthesize IgM cells in the bursa of Fabricius which later disseminate into the thymus and other lymphoid tissues and organs during prenatal development in chicken (Kincade and Cooper, 1971; Van Alten and Meuwissen, 1972; Islam *et al*., 2013). Therefore, the low levels of IgM-positive lymphocytes in the embryonic and early post-hatch chicks' thymus may have resulted from migration of limited number of IgM-positive cells from the bursa to the thymus at these stages of development.

However, in terms of time of appearance of IgM-positive lymphocytes in the thymus, varied opinions have been

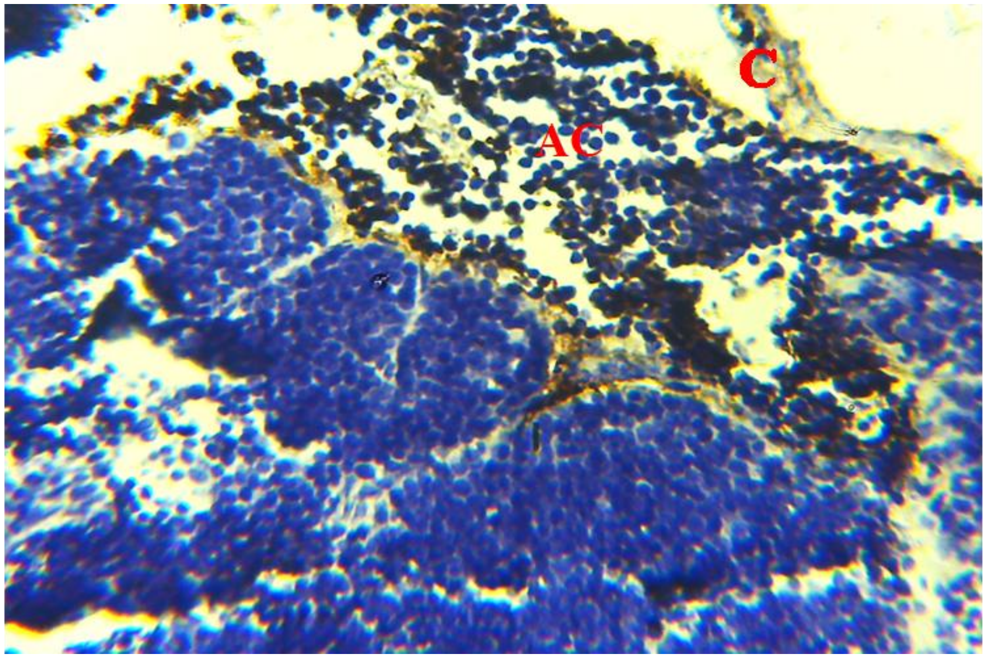

Figure 5. Photomicrographs of immunostained section of the thymus at D 14 post-hatch. Note the accumulation of IgM-positive lymphocytes, AC in the subcapsular region. C: capsule; X400.

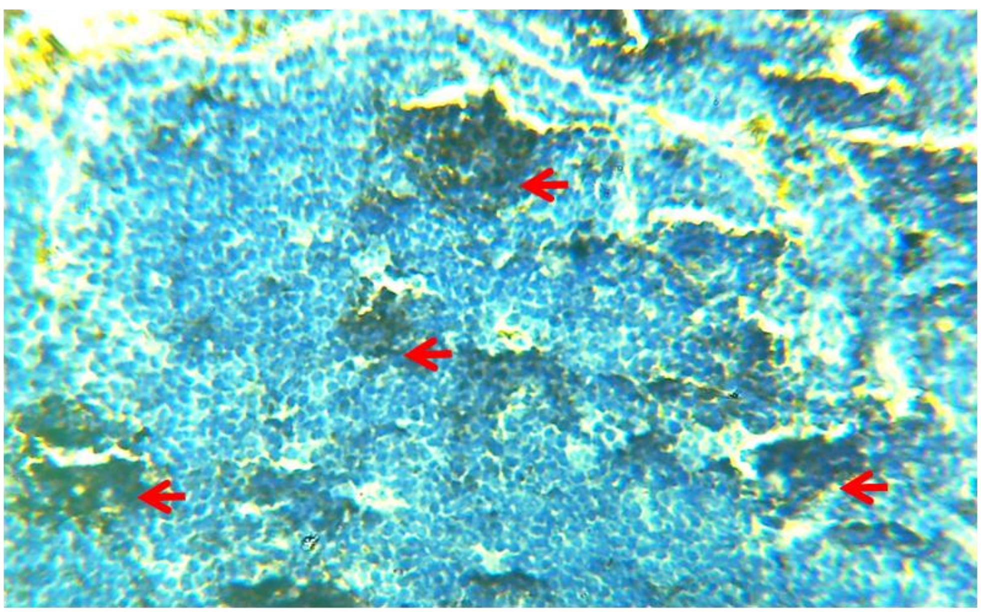

Figure 6. Photomicrographs of immunostained section of the thymus at D 28 post-hatch showing the parenchyma. Note the clusters (arrow) of IgM-positive lymphocytes; X400.

documented. Yamamoto *et al*. (1977) reported that no IgM-positive lymphocytes were expressed in the thymus of chicken embryos; rather the bursa of Fabricius only synthesized IgM-positive lymphocytes after hatching. Kincade and Cooper (1977) observed that, IgM-positive lymphocytes first appeared in the chicken thymus at EID 20 as reported by Islam *et al*. (2013).

Further results showed that there was a steady increase in the magnitude of occurrence of IgM-positive lymphocytes between post-hatch D 14 and D 42. This progressive increase in the quantity of IgM-positive lymphocytes may have resulted from increased exposure of the chicks to environmental antigens that may have triggered more intensive immunological reactions that led

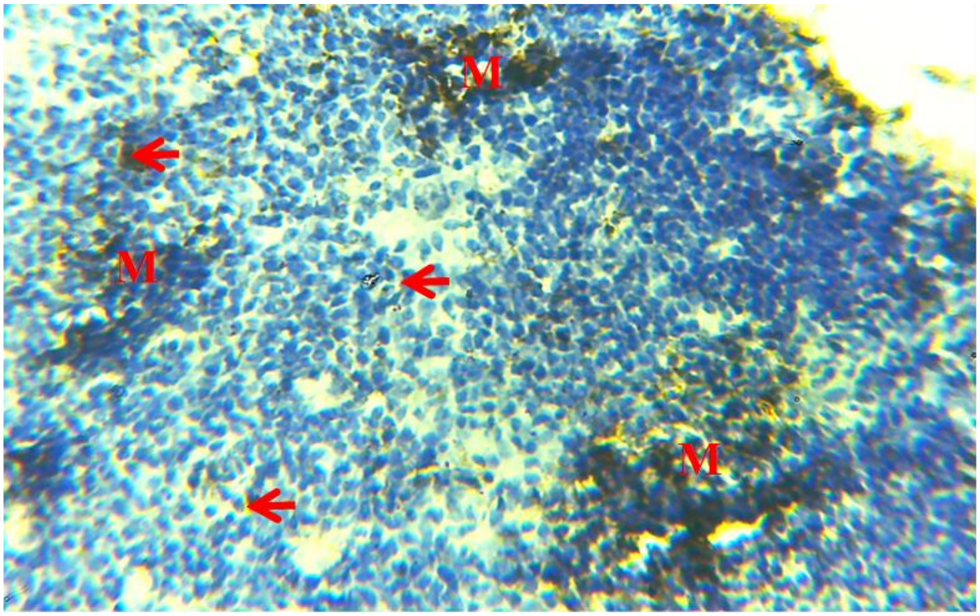

Figure 7. Photomicrographs of immunostained section of the thymus at D 42 post-hatch. Note the masses, M and diffusely distributed (arrow) IgM-positive lymphocytes in the thymic parenchyma; X400.

to the proliferation of IgM-positive cells. Masum *et al*. (2014) observed a similar increase in the quantity of IgM-positive lymphocytes in broiler chicks vaccinated against Newcastle disease. According to these authors, IgM-positive cells recorded significant values in the thymus at D 14 and D 28 following inoculation with Newcastle disease vaccine. The similarity in age and pattern of expression of IgM-positive lymphocytes between vaccinated broiler chicks and unvaccinated Nigerian native chicks further proves the strong innate humoral immunological potentials associated with the Nigerian native chicken. As previously stated, the thymus is composed mainly of T-lymphocytes that are not antibody synthesis oriented, but Masum *et al*. (2014) reported that the existence of high levels of IgM-positive lymphocytes in the thymus of the vaccinated chickens is a result of B-lymphocyte infiltration into the thymus via blood circulation and differentiation into Ig-containing plasma cells for local defensive functions.

In conclusion, IgM-positive lymphocytes appeared in the thymus as early as EID 10, and the presence of appreciable quantities of IgM-positive cells in the post-hatch thymus, irrespective of the naturally low densities of B lymphocytes in the thymus, indicates high humoral immunological tendencies in the Nigerian native breed of chicken.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest among the authors.

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