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Immunophenotyping Panel of T Lymphocyte in Water Buffalo Spleen (*Bubalus bubalis*) and its Significant Correlation to Ruminant Diseases

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ABSTRACT

Spleen occupies an effective immunological role in mammals especially economic animals like water buffalo. Regardless of that, not literature points attention to immune cells homing buffalo spleen. This study sheds light on the distributional panel of T lymphocyte in water buffalo as a framework to easily diagnose any alteration in spleen composition that may refer to immunological disorders. Twenty spleens of both sexes, 3.0 ±0.5 years and 400.0 ±50.0 kgs were selected randomly from apparently healthy animals. Tissues were subjected to T cell antibodies via applying immunohistochemistry staining protocol. Quantitative analysis of the distribution of these antibodies was assessed by image J program. Area percentage of each antibody was evaluated by aid of R- program. CD3 was expressed for all T lymphocytes, while CD4, CD8, and CD45RO referred to a helper, cytotoxic, and memory T cells, respectively. Expression of $CD3^+$, $CD4^+$, and $CD8^+$ cells was high in the periarterial lymphatic sheath, red pulp, and marginal zone with a significant area % difference than lymph nodule. Unlikely, $CD45RO^+$ expression gained insignificant area %. Most likely the obtained findings will give a reference scheme for T lymphocyte in buffalo spleen. This research presented a quantitative analysis to the distribution of distinct types of T lymphocyte in normal splenic tissue of water buffalo species.

Introduction

Water buffaloes stand for a vital contribution to animal production in many countries. They mark a potential source for dairy, meat products, moreover their sharing also in industrial ones. Water buffaloes have unique over stranding to the bad environmental conditions and despite that, they produce effectively. Disease resistance, the power to adapt in varied climate conditions, giant edibleness of poor pasture, and quickly gain in body weight display their efficient performance which must be utilized (Rashad *et al*., 2020; 2021).

The mammalian immune system consists of specialized lymphoid tissues, plus an enormous network of lymphatic vessels storing tissue lymph and cells in virtually every part of the body, and a reservoir of lymphoid cells continually recirculating between the lymphatic system and the bloodstream. The lymphoid organs categorize as primary lymphoid organs: bone marrow, thymus, and the patches in the intestine of the Peyer in ruminants. Cells are fashioned in the primary lymphoid organs enter the lymphocyte recirculating pool and fill the spleen, lymph nodes, and secondary lymphoid organs. Spleen in bovine is present as an encapsulated organ composed of two main parts: white pulp (WP) and red pulp (RP) (Schneder *et al*., 2011).

Immunohistochemistry makes a thorough analysis for the distribution of antigens in the tissue and the spatial relationship between the cells that express them and other structures of cells and tissues. Nevertheless, healthy tissue morphology and antigenicity are prerequisites. The marker panel eases the proof of identity, by immunohistochemistry antibodies to find distinct types of white blood cells, at dissimilar stages of maturity and functionally important molecules on cell surfaces (González *et al*., 2001).

The recognition among types of cells is deprived by the limited availability of anti-bovine antibodies and antigen recovery therapies. Accordingly, previous studies depend mainly on antibodies that have a cross reactivity to bovine species, if no specific antibodies are available (Niku *et al*., 2006). The significant advances in these methodologies enable the identification of multiple immune parameters, as well as an efficient tool for measuring the spread and quantity of certain cells through immune populations. Furthermore, it is conceived as a representative tool in understanding immune cell involve-

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ment, concerning ruminant illness pathophysiology, which gives greater flexibility in routine diagnosis (Keresztes *et al*., 1996).

The spleen of water buffalo is still poorly defined in terms of its immune cells and its adequate role in different diseases. Therefore, research in this study was pointed to recognize the distribution of the T lymphocyte as one of the immunocompetent cells in water buffalo's spleen by using immunohistochemical methods, the authors hope that the findings open the insight to immune response background to some buffalo diseases.

Materials and methods

Sample Collection

The splenic tissues managed in this investigation were gathered from the Munib Municipal Abattoir, Giza Government, Egypt. A total of 20 spleens, weighing 400.0±50.0 kilo grams were selected from adult apparently healthy slaughtered water buffalos of each sex (10 Male and 10 Female) and 3.0±0.5 years old, which did not reveal any systematic signs nor symptoms of sickness. The samples were randomly collected and transported through ice packs to the veterinary histology laboratory, Cairo University, Egypt. The samples were labeled promptly, that was bearing a code range for later identity. Each sample gently washed in regulator water on a dissection receptacle. The fixation was performed using 10% formalin. Samples were dehydrated by using an ethanol-xylol collection and then embedded in paraffin. Sections of paraffin (4-6 µm) were stained by routine method of peroxidase- anti peroxidase (PAP) (Petrosyan *et al*., 2002).

Staining Protocol

Splenic tissue sections were exposed to deparaffinization and rehydration steps. Then, by using a buffer, sections were washed twice. Some antibodies required incubation of splenic tissue in a digestive enzyme according to kit guidelines. Then, sections were washed four times using a buffer. To lack non-appreciate background staining owing to endogenous peroxidase, slides were incubated in Hydrogen Peroxide Block for ten minutes, after that slides were washed two times for 5 min in TBS plus 0.025% Triton X-100 with gentle agitation. The splenic slides were kept until draining for a few seconds and then were wiped with tissue paper. Each primary antibody was diluted to the manufacturer's recommendations and then applied to splenic slides. In this study, the primary antibody distinguished as monoclonal and Mouse Anti Human/Bovine (cross-reactivity) and from 'Thermo Fisher Scientific' (vendor). They existed in CD3 (F7.2.38) (Cat# MA1-21199, RRID: AB_558171), CD4 (4B12) (Cat# MA5-12259, RRID: AB_10989399), CD8 (C8/144B) (Cat# A500-021A, RRID: AB_2773818) and CD45 (2B11+ PD7/26/16), (Cat# MA1-82612, RRID:AB_928414). Then incubation (Time of incubation occurred to each antibody from protocol designed by the manufacturer), washed four times in buffer. The primary antibody enhancer was added at room temperature, incubation was performed for ten minutes and washing was repeated for four times in buffer. Horseradish peroxidase polymer (HRP Polymer) was added at room temperature, incubation went ahead for 15 minutes, then splenic sections were washed four times in buffer. One drop of (40µl) 3,3' Diaminobenzidine (DAB) Plus Chromogen were added to 2ml of DAB Plus Substrate then were mixed by swirling, applied to slide and incubated for five minutes. The last wash was done by using distilled water for four times. After counterstaining, coverslip was applied by the help of permanent mounting media. Negative control of splenic tissue sections was raised beneath the equivalent conditions with low amount of a primary antibody (Gnanadevi *et al*., 2019). Microscopic observations achieved through Leica microscope (CH9435 Hee56rbrugg) underneath different magnification.

Evaluation of immunohistochemical results "Area Percentage" (Specific area/ Antibody)

Cell numbers were calculated as the number of cells per cross-sectional area using the cell counter/ color intensity/ color threshold features and IHC plugin of Image J program (Schneider *et al*., 2012), and then was used for evaluating area percentage (Area%) for each antibody in spleen compartments as white pulp (WP), marginal zone (MZ), and red pulp (RP).

Statistical analysis

Data related to area% presented as Mean and Standard Error "SE". Variance and multiple comparison tests; ANOVA performed using R codes and Shapiro-Wilk normality test for measuring P-value (R Core Team, 2013). This package is available at CRAN (https://cran.r-project.org/). P-values in a statistic that is less than 0.05 stood for a significant result.

Results

Immunohistochemistry

T-lymphocytes were classified according to their receptors and functions into three categories: T-helper, T-cytotoxic, and T-memory cells.

Almost all types of T-lymphocytes in the spleen of water buffalo showed strong positive immunoreactivity to the CD3 antibody. This immune expression was observed in all compartments of buffalo spleen but with differences in the distribution (Fig. 1A). The highest distribution of the T-lymphocyte cells that positively reacted with the CD3 antibody was noticed in the periarterial lymphatic sheath (PALS) (Figs.1B &1C). Meanwhile, the lymph nodules showed the least distribution. Moderate distribution was demonstrated in MZ (Fig.1D) and high expression was observed in RP areas (Fig.1E).

A special antibody called CD4 identified helper T- lymphocytes (Th). In the general demonstration of the buffalo spleen, T-helper cells reacted positively to the CD4 antibody in all compartments. The highest distribution of $CD4^+$ Th cells was detected in MZ and RP, while these cells in the PALS and lymph nodules showed low distribution (Fig. 2A). Near to the central arteriole, Th cells reacted positively to CD4 (Fig. 2B). The surface expression distinguished in two patterns along the surface of Th cells; the first appear light and small reaction while the other highlighted in intense and irregular form. Inside lymph nodule and PALS of buffalo spleen, $CD4^+$ expression was visualized along the surface of Th cells (Figs. 2C& 2D). On the other hand, a high distribution of surface expression CD4+Th cells was clarified in MZ (Fig. 2E) and RP areas (Fig. 2F).

CD8 antibody used to highlight the cytotoxic T-lymphocytes inside the spleen of water buffalo representing the high distribution of this cell type in the red pulp and marginal zone than the lymph nodule area. In splenic lymph nodule of water buffalo, the expression of the CD8 antibody was observed on the surface of T-cytotoxic cells (Fig. 3A). Most of the cytotoxic T-lymphocytes' expression was scattered in the MZ area (Fig. 3B). In the RP, expression was noticed either round regular or scattered on cytotoxic T-lymphocytes' surface (Fig. 3C).

Another important type of T-lymphocytes was memory T-

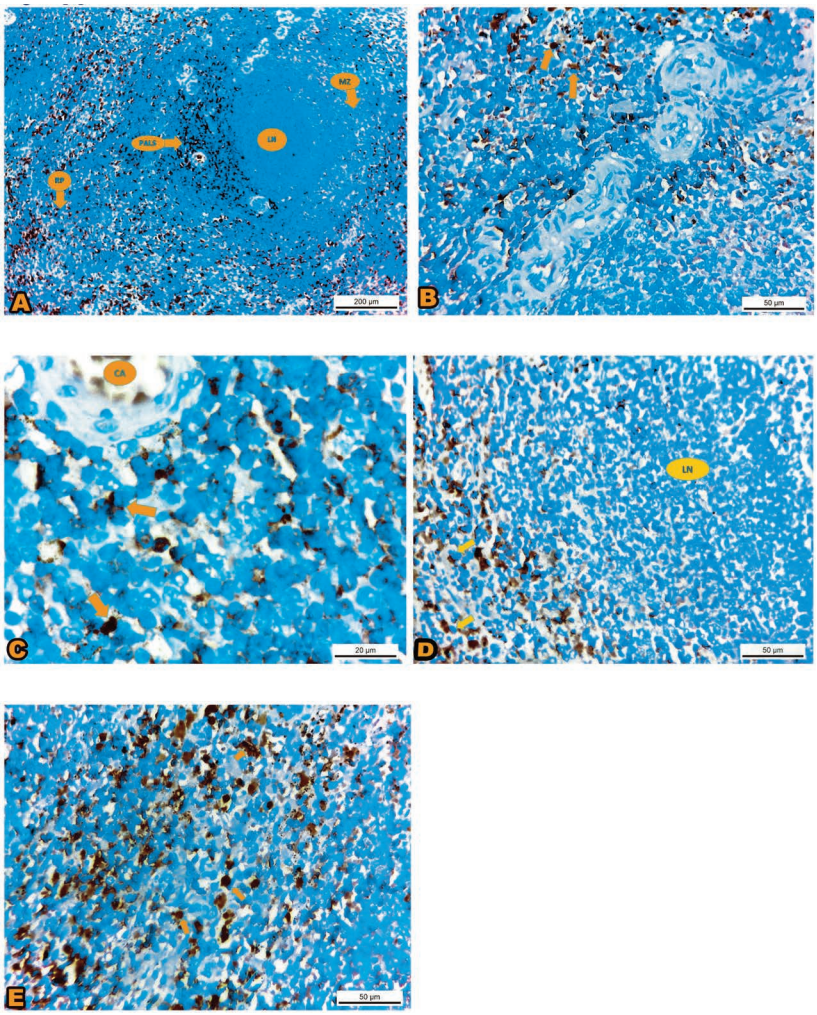

Fig. 1. Photomicrograph of water buffalo spleen demonstrated the reaction of T-lymphocytes to the CD3 antibody and showed: (A) High distribution of the $CD3^+$ T-lymphocytes in the "PALS", least distribution inside the lymph nodules "L.N." and moderate one was noticed in the marginal zone "MZ" and red pulp "RP". (CD3 X100). (B) Highest distribution of the $CD3^+$ T-lymphocytes (arrows) in the PALS. (CD3 X400). (C) $CD3^+$ T-lymphocytes with high immune-positive expression (arrows) in the PALS around central arteriole (CA). (CD3 X1000). (D) Marginal Zone clarified the $CD3^+$ T-lymphocytes with moderate expression (arrows). Notice: least distribution in the lymph nodule (LN) (CD3 X400). (E) Moderate $CD3^+$ T-lymphocytes reaction in the red pulp along splenic cords (arrows) (CD3 X400).

cells. The antibody used to highlight its distribution was CD45RO. The surface expression of CD45RO+ T-memory cells was recognized in LN and MZ in lesser distribution (Figs. 3D&3E) than that of the RP area (Fig. 3F).

Evaluation of immunohistochemical results

ANOVA test showed a significant difference in the area % values of T-lymphocytes by using CD3, CD4 &CD8 antibodies, and no significant difference was noticed by using the CD45RO antibody when comparing the splenic areas (Fig. 4 and Table 1).

Table1. Area% values of T Lymphocytes Antibodies.

	WP	MZ	RP	*P Value*
CD3	4.11±0.2^c	8.44±0.55^b	22.27±3.5^a	0.006*
CD4	2.67±0.59^c	5.13±0.83^b	20.92±2.21^a	0.02*
CD8	3.23±0.32^c	3.82±1.19^b	18.63±4.5^a	0.009*
CD45RO	2.80±0.30^c	6.20±0.75^b	16.77±2.66^a	0.06

Data were expressed as Mean ± Standard Error. *P- Values ≤ 0.05: significant. WP: White pulp; MZ: Marginal zone; RP: Red pulp.

Discussion

The cell population of the spleen has a design not only to defend against acute infection but also is involved in a specific immune response that enhances immunity in bovine animals (Goff *et al.*, 2010). There seems to be a rising knowledge of the great significance of lymphocytes with innate activities in the homeostasis of the tissue, immune modulation, and prevention of infections (Zhang, 2013). The variations that arise in groups of splenic lymphocytes concerning exact location, structure, and cellular modification have important implications for immune responsiveness after certain diseases (Zhang *et al.*, 2017).

The current investigation proved that all forms of T lymphocytes showed strong positive immunoreactivity to the CD3 antibody in the spleen of water buffalo. On the contrary, in yak spleens, some CD3 positive cells were present (Huang *et al.*, 2019). Findings from this study reflected the immune expression in all compartments of buffalo spleen but with differences in the distribution. PALS showed the highest distribution of $CD3^+$ T lymphocyte while the least distribution of $CD3^+$ T-lymphocytes in the obtained result remarked lymph nodule (LN) area. On the other hand, the reaction was moderate in MZ and high in the RP. The explanation of the pervious findings declares LN to B-cell dependent areas, low amount of $CD3^+$ T cells in the centers of LN, which may serve as a regulator cell (Zidan *et al.*, 2000).

Concerning their allocation inside T cells, along with the PALS, MZ, and RP, $CD3^+$ T-lymphocytes spotted primarily as

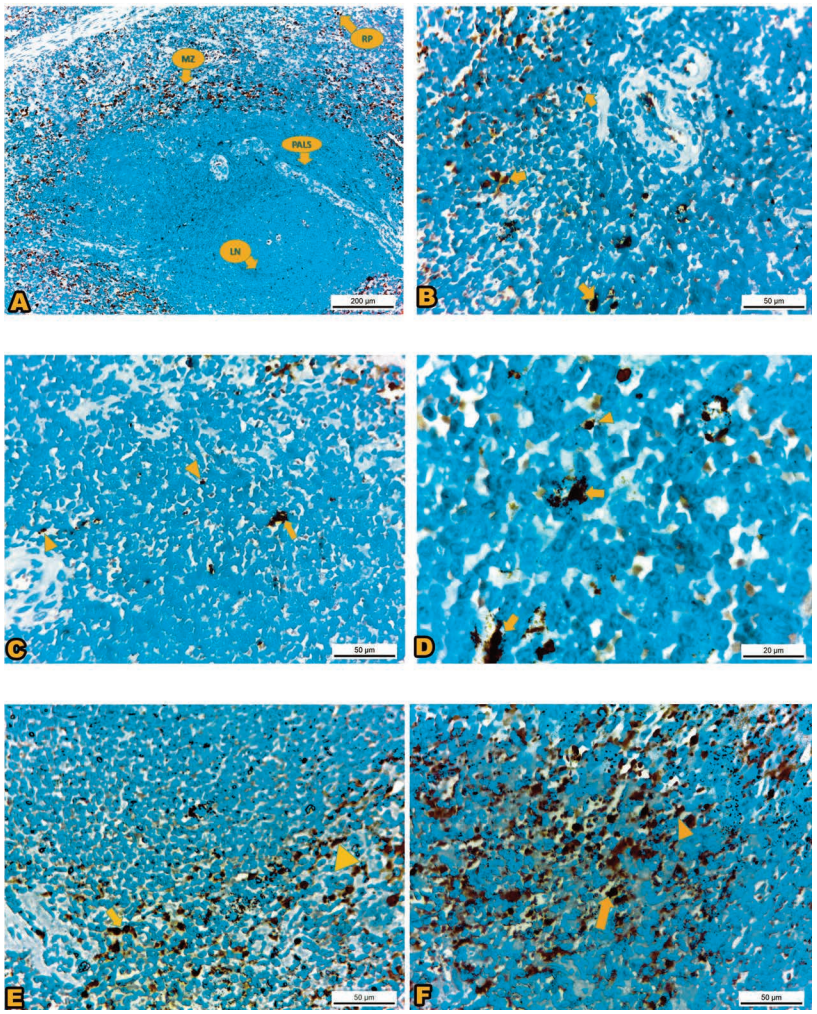

Fig. 2. Photomicrograph in water buffalo spleen outlined the reaction of T-helper cells to the CD4 antibody and showed: (A) High distribution of the Th cells in the marginal zone (MZ) and the red pulp (RP) and low distribution in the "PALS" and lymph nodule (LN). (CD4 X100). (B) Positive immunoreaction of the Th cells around the central arteriole (arrows). (CD4 X400). (C) $CD4^+$ Th cells inside the LN expressed as surface light (arrowhead) or intense (arrow) reaction. (CD4 X400). (D) PALS with $CD4^+$ Th cells expressed in light pattern (arrowhead) in some cells and intense (arrows) in other cells. (CD4 X1000). (E) High distribution of $CD4^+$ Th cells in the MZ mostly expressed as intense reaction (arrow). Notice: $CD4^+$ as light flat expressed cells (arrowhead). (CD4 X400). (F) Red pulp clarified highly distributed CD4+ Th cells which seen mostly with intense expression (arrow) or few light ones (arrow). (CD4 X400).

surface expression. The present study reinforces the essentiality of a CD3 antibody for the transmission of antigen-recognition signals into the T-cell cytoplasm and the control of the T cell activation by antigen receptor (TCR) complex's cell surface expression. Moreover, the involvement of CD3 in growth arrest, cell survival, and proliferation are trigger by TCR (Kuhns *et al*., 2006).

A special antibody called CD4 detect helper T- lymphocytes (Th). In this study, general demonstration of the buffalo spleen revealed that T-helper cells responded positively to the CD4 antibody in all partitions. Specifically, $CD4^+$ Th cells in the PALS and LN have taken the low distribution. Around the central arteriole, LN, and PALS of buffalo spleen, CD4+ Th cells acted in diverse ways: light, or intense in their reaction. In parallel to the results mentioned by González *et al*. (2001) in sheep. Accordingly, the obtained results suggest the integration of T-lymphocytes in improving the requisite collaboration between B-lymphocytes and T-lymphocytes at germinal centers (Abdel-Magied *et al*., 2001).

Conversely, data of the current study revealed the highest distribution of CD4+ Th cells in MZ and RP. Their expressions exist in a surface form. However, in bovine animals, Zhang *et al*. (2017) recognized a few positive CD4+ Th cells in the RP. Unlike Huang *et al*. (2019) whose results in healthy yaks were familiar with this study. The present study supports the postulations in the importance of this cell inside water buffalo spleen as natural regulatory T cells (n T regs), defined as CD4+ a hallmark of T helper cell (Sakaguchi *et al*., 2008). The reason for that lies in the involvement of CD4+ Th cells in cytotoxic T-cells growth and its action, management of B cell activities, and phagocyte stimulation. CD4 molecules claim to be chemotactic factor IL-16 receptor and as a proinflammatory cytokine achieving a prominent level of inositol triphosphate, calcium, and p56lck autophosphorylation (Lynch *et al*., 2003). They regulate cellular immunity through their embracing in the identification of major histocompatibility complex (MHC) molecules on the cell surface (Ossendorp *et al*., 2000) and their capacity to modulate the migration of NK cells. As a result of its importance in T independent immune function, any defect in CD4 will cause impairment in the response of B cells to the Escherichia coli lipopolysaccharide (LPS) of E. coli (Elkins *et al*., 1991).

Reports assumed by Kim *et al*. (2008) in mammals, proved that positive expression of CD4 in the follicular dendritic cells along with LN, MZ, and RP. Sometimes, $CD4^+$ follicular dendritic cells associated with $CD4^+$ Th-lymphocytes that indicated the critical role of this receptor to initiate a certain immune response.

The CD8 antigen is a cell surface glycoprotein found on all cytotoxic T lymphocytes. CD8 focusses on the cytotoxic T-

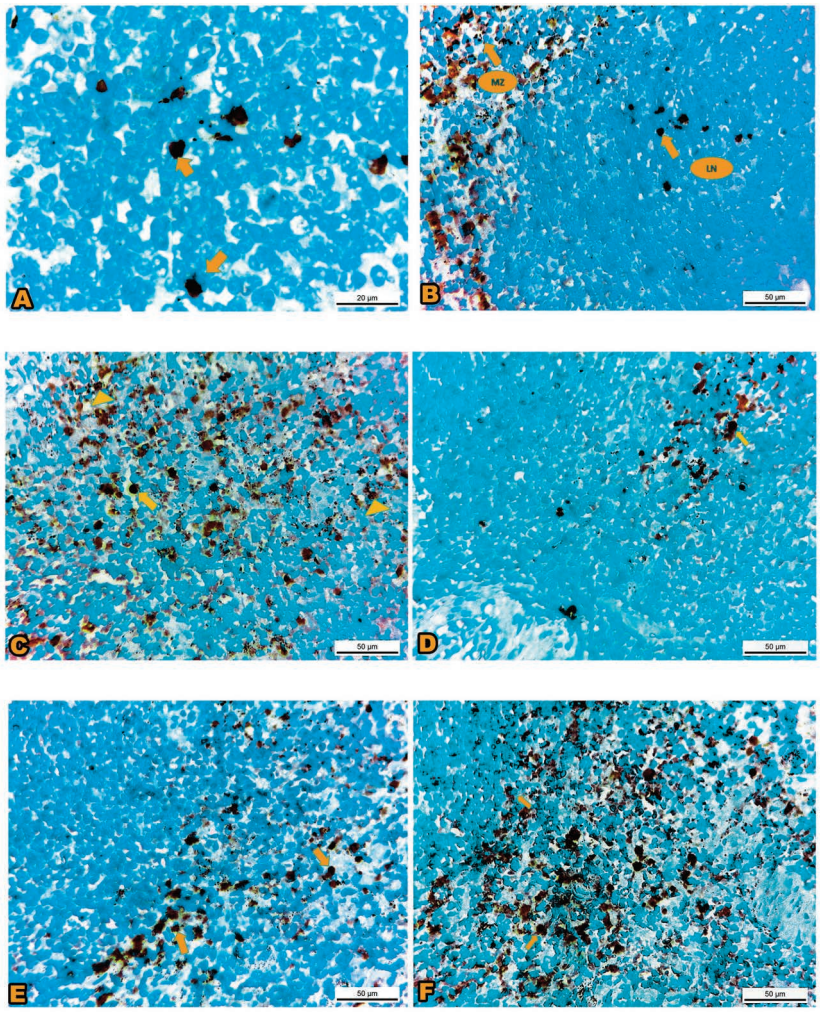

Fig. 3. Photomicrograph of water buffalo spleen demonstrated: (A:C) The reaction of T-cytotoxic cells to CD8 antibody and showed: (A) Splenic LN presented the surface immunoreaction (arrows) of $CD8^+$ T-cytotoxic cells. (CD8 X1000). (B) $CD8^+$ T-cytotoxic cells remarked in the marginal zone (MZ) area in scattered manner. Notice: the surface expression of these cells inside lymph nodule (L.N.). (CD8 X400). (C) Splenic RP highlighted $CD8^+$ T-cytotoxic cells with their regular expression (arrow) in some cells or scattered in other cells (arrow heads). (CD8 X400). (D: F) The reaction of T-memory cells to CD45RO antibody and showed: (D) CD45RO+ memory T-cells were less expressed inside LN as immunopositively reaction (arrow). (CD45RO X400). (E) Marginal zone presented the less CD45RO+ memory T-cells expression along their surface (arrows). (CD45RO X400). (F) High distribution of CD45RO+ memory T lymphocytes in the RP clarified the surface expression (arrows). (CD45RO x400).

lymphocytes inside the spleen of water buffalo being the high distribution of this cell type in RP and MZ than the LN area. Results from this study are coincidental with the reports of Schneder *et al.* (2011) in bovine animals. It confirms the adjusting ability to the exposed areas where the antigen is supposed to be trapped externally. CD8 is essential to ensure the intact architecture of the spleen (Borch *et al.*, 2019).

Thoroughly, inside the LN area of water buffalo spleen, few $CD8^+$ T-cytotoxic cells seen in this study. The expression highlighted as surface immunoreaction on T-cytotoxic cells. Authors of this work support the suggestion of Unsoeld *et al.* (2004) who explained the downregulation potency of CD8+ effector T cells to release specific chemokines. Thus, it leads to their omission from the splenic white pulp, enabling these cells to access blood and peripheral tissues. Downregulation of CD8+ effector T cell expression allowed an efficient antiviral action and hence can suppress the development of Bovine leukemia virus (BLV) growth inside B-cells (Okada *et al.*, 1993).

The majority of cytotoxic T lymphocyte in the marginal zone area proved their expression on the surface in a scattered manner. Besides, RP expression to CD8+ T-cytotoxic featured either round regular or scattered form in this literature. The main chief role of CD8 in these areas points on the CD8 antigen is considered a coreceptor on the T lymphocyte permitting the identity of any antigens (protein or non-protein origin) presented by antigen-presenting cell regarding class I MHC molecules (Born *et al.*, 2013). Consequently, CD8+ T cells assemble efficient immunity during cell to cell interaction (Antibodies-online, 2019).

Cytotoxic T lymphocyte lists a specific lymphocyte population in blood, tissues, and inflammation sites. A high percentage of ruminant T lymphocyte express CD8 have various defensive functions in the immune system of ruminants (Maupome, 2019). Recent literature conducted to estimate the differentiation of T cell. The judgment of immune infiltrates sequences highlights the relation between type of cell and their function in bovine animals (Swaminathan *et al.*, 1999) predicts and diagnose defects following enteropathogenic Escherichia coli, many types of cancers and TB disease. Therefore, early-stage cell density quantification of CD3 + and CD8 + T cells has a critical impact on the prognosis of the disease (Herbst *et al.*, 2014).

This investigation used CD45RO to spotlight memory T lymphocyte distribution inside splenic tissue of water buffalo. The surface expression of $CD45RO^+$ T-memory cells remarked LN and MZ areas in lesser distribution than that of the RP area. Related inspections evaluated in cattle by Keresztes *et al.* (1996). In agreement with Bembridge *et al.* (1995) in cattle

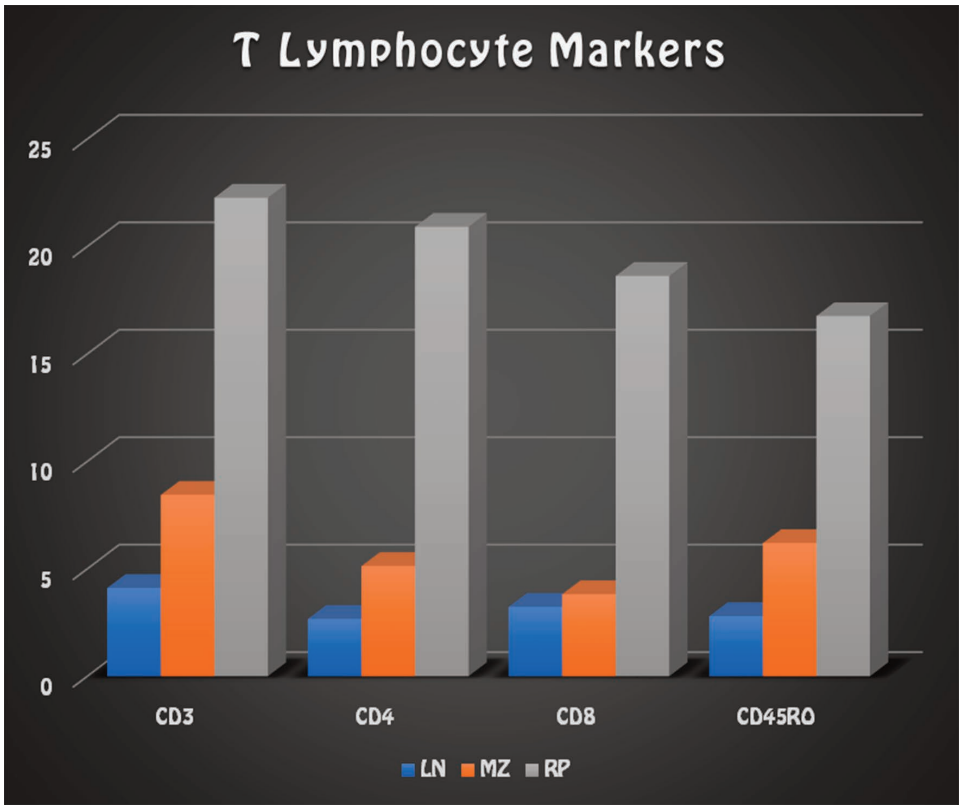

Fig. 4. Area % values of T- Lymphocytes.

who clarified that $CD4^+$ T cells can be categorized into $CD45RO^+$ and $CD45RO^-$ (naïve) subpopulations. They added that the two types generated IL-2 mRNA, meanwhile, $CD45RO^+$ emitted plenty of quantities of IL-4 as well as IFN-y mRNA. At that time, naïve T cells exposed to a stimulus, they converted to $CD45RO^+$ along with responsibility in the memory Treg pool (Booth *et al*., 2010). Authors of this work support data focused on the relation between proliferating T cells and Mycobacteria Bovis in infected cattle, so priority must be given to CD45RO antigen in the detection of Mycobacteria Bovis as discussed by Maupome (2019).

Conclusion

The results obtained in this study spot line on water buffalo spleen regarding the distribution of each T cell type that conducted effective roles and demand more investigation. Further insight was on contributions of several receptors in disease diagnosis as Bovine leukemia virus, Mycobacterium bovis, different forms of cancer, and pathogens like enteropathogenic Escherichia coli. Such meaningful results aid in the induction of effective immunological memory and represented potential new targets in vaccination strategies.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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