Blood lymphocyte subpopulations in healthy water buffaloes (*Bubalus bubalis*, Mediterranean lineage): reference intervals and influence of age and ~~lactation phase~~reproductive history

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Abstract

There is an increasing interest toward infectious diseases and mechanisms of immune response of water buffaloes, mainly because of the growing economic impact of this species and of its high-quality milk. However, little is known about the immune system of these animals in physiological conditions.

Recently, a wide number of antibodies ~~cross-reacting~~cross reacting with buffal~~o~~o~~ine~~ antigens has been validated for use in flow cytometry (FC), allowing detailed characterization of the lymphocytic population in this species.

The aim of the present study was to describe the lymphocyte subpopulations in a large number of healthy water buffaloes, providing reference intervals (RIs), and to assess whether the composition of blood lymphocyte population significantly varied with age and ~~lactation phase~~reproductive history. Our final aim was to lay the ground for future studies evaluating the role of host immune response in water buffaloes.

One-hundred-twelve healthy buffaloes from four different herds in the South of Italy were included in the study. All animals had been vaccinated for Infectious Bovine Rhinotracheitis (IBR), Salmonellosis, Colibacillosis and Clostridiosis, and all herds were certified Brucellosis- and Tuberculosis-free. Venous blood collected into EDTA tubes was processed for FC, and the percentage of cells staining positive for the following antibodies was recorded: CD3, CD4, CD8, CD21, TCR-δ-N24, WC1-N2, WC1-N3 and WC1-N4. Absolute concentration~~s~~ of each lymphoid subclass was then calculated, based on automated White Blood Cell (WBC) Count. Reference Intervals were calculated according to official guidelines and are listed in the manuscript.

The ~~concentration~~ composition of ~~each~~ the lymphocyte ~~sub~~population varied with age and reproductive history, with animals <2-years-old and heifers having higher concentration of most of the subclasses. ~~On the contrary, lactation phase had minimal influence on the composition of the lymphocytic population~~.

The present study provides RIs for the main lymphocytic subclasses in healthy water buffaloes, highlighting gross differences between young and old animals. Establishment of age-specific RIs is recommended in water buffaloes. The data we present may ~~serve~~ be useful as a basis for further studies concerning mechanisms of immune response toward infectious agents in water buffaloes.

Keyword: water buffaloes; lymphocytes; flow cytometry; reference intervals

Introduction

Water Buffalo has been domesticated thousands of years ago in the Asian region (Groeneveld et al., 2010). Today, ~~livestock~~ farming of water buffaloes, either of the swamp or the river subtype, has spread worldwide and is gaining interest because of the high quality and nutritional value of buffalo milk compared to cow milk (Pasquini et al., 2018). Indeed, cow milk is also used for illicit adulteration of buffalo milk (Durakli Velioglu et al., 2017; Galan-Malo et al., 2018).

With the growing economic impact of buffalo, the prevalence of infectious diseases affecting this species and the mechanisms of host immune response are in the spotlight of veterinary research on farm animals (Panigrahi et al., 2016; El-Halawany et al., 2017, Hornok et al., 2018; Martins et al., 2018; Paradiso et al., 2018). Still, little is known about the immune system of healthy water buffaloes. In 2016, De Matteis and colleagues used a multi-technique approach to highlight the possible role of Leptin Receptor (LEPR) in the immune response of water buffalo (De Matteis et al., 2016). No antibody ~~cross-reacting~~cross-reacting ~~against~~ with buffalo LEPR was commercially available: thus, authors tested two different anti-human antibodies and finally selected a polyclonal rabbit antibody to test LEPR expression in blood cells of water buffalo by flow cytometry (FC) and confocal microscopy. Leukocytes subpopulations were identified using anti-bovine antibodies. One year later, another study tested a wide number of antibodies for their cross-reactivity with buffalo antigens (Grandoni et al., 2017). The authors also report the percentages of cells expressing some of the antigens tested, revealing differences between young and old animals. Despite these interesting data, only 25 animals were included in the study.

The aim of the present study was to describe the lymphocyte subpopulations in a large number of healthy water buffaloes, providing reference intervals (RIs), and to assess whether the composition of blood lymphocyte population significantly varied with age and ~~lactation phase~~reproductive history. Our final aim was to lay the ground for future studies evaluating the role of host immune response in water buffaloes.

Materials and methods

Animals

Mediterranean buffaloes (MB) were randomly selected during the routine clinical activities of the Herd Health Management Service of the Veterinary Teaching Hospital - Department of Veterinary Medicine and Animals Production of Napoli (Italy) from four different herds in the South of Italy between January and September 2018. All four herds were certified Brucellosis- and Tuberculosis-free and all animals had been vaccinated for Infectious Bovine Rhinotracheitis (IBR), Salmonellosis, Colibacillosis and Clostridiosis. ~~All the~~ MB were considered healthy if they did not show~~n~~ any clinical sign consistent with infectious or metabolic diseases ~~with~~in the two months prior to sampling. All the procedures performed in this study followed the common good clinical practices and received an institutional approval by Ethical Animal Care and Use Committee of University of Naples Federico II (PG/2017/0099607); moreover, the farmers were previously informed and in agreement with purpose and methods used.

Flow cytometry

Peripheral blood (PB) samples were collected from the coccygeal vein into Vacutainer tubes containing EDTA, shipped over-night to the FC laboratory and processed within 24 hours.

Prior to labelling for FC, a complete blood count (CBC) was performed with an automated hematology analyzer equipped with a veterinary software (Sysmex XT 2000-iV, Sysmex, Kobe, Japan). White blood cells (WBC) are displayed in a scattergram according to their light scatter and fluorescence signal produced by staining nucleic acids with a polymethine dye (DIFF-channel) (van der Meer et al., 2002) and WBC differential is provided by gates preset on the scattergram. As none of the gates provided by the software fitted with water buffalo samples, new gates were created manually for this species and all samples were analyzed with these gates (Figure 1A).

For each sample, a PB smear was prepared and stained with May-Grünwald Giemsa stain. A 100-cells differential count was performed to validate the WBC differential provided by the software.

Different volumes of PB were put in FC tubes, according to the WBC count of each animal, in order to have $5x10^5$ nucleated cells/tube. ~~In order t~~To avoid ~~a~~non-specific antibody binding, 25 µl of RPMI

1640 solution containing 5% fetal bovine serum (FBS) and 0.2% sodium azide were added to each tube. Samples were incubated for 20 minutes at room temperature with the following primary antibodies: anti-CD3 (clone MM1A), anti-CD4 (clone CACT138A), anti-CD8 (clone CACT80C), anti-CD21 (clone GB25A), anti-TCR-δ-N24 (clone GB21A), anti-WC1-N2 (clone BAQ4A), anti-WC1-N3 (clone CACTB32A) and anti-WC1-N4 (clone BAQ89A) (Grandoni et al., 2017). All primary antibodies were provided by Washington State University (Pullman, WA) and had been tittered before use to select the best working dilution. Thereafter, samples were washed twice in PBS 1x. Three µl of FITC-conjugated secondary antibody (goat anti-mouse Ig, Becton Dickinson, San Josè, CA) were added to each tube, including the negative control (unstained cells). After 20 minutes, 2 ml of a solution containing 8% ammonium chloridre were added to each tube to lyse erythrocytes. Cells were then washed twice and finally suspended in 500µl of PBS 1x.

All samples were acquired with a FACScalibur flow cytometer and analyzed with CellQuest software (Becton Dickinson, San Josè, CA). For each animal, a gate was set in the morphological scattergram to include the lymphocytic population (small-medium cells with low complexity index) and the percentage of lymphoid cells out of total nucleated cells was recorded. Thereafter, the percentage of antibody-positive cells out of lymphoid cells was recorded for each antigen tested. Absolute concentration of each cellular subpopulation was finally calculated, based on the WBC count and the percentages obtained via FC.

Only percentages obtained by FC were used in the present study. Results from automated and manual leukocytes differential count only served as a quality control for FC and both were performed to exclude that gross cell loss happened during sample processing for FC and washing steps.

Statistical analysis

RIs were calculated with the Reference Value Advisor version 2.1 (Geffré et al., 2011), according to the ASVCP official guidelines (Friedrichs et al., 2012). None of the investigated variables had a Gaussian distribution or could be transformed to. Thus, results from robust methods were considered

as the most appropriate, also according to the color-code provided by Reference Value Advisor (Geffré et al., 2011).

For statistical aims, animals were arbitrarily divided into 5 groups based on age (<2 years, 2-4 years, 4-6 years, 6-8 years, > 8 years). Animals aged >2 years were then subdivided ~~and~~ into ~~other~~ 3~~4~~ groups based on ~~the number of days in milk (DIM) (<45 DIM, 45-200 DIM, 200-300 DIM, >300 DIM)~~reproductive history (heifers, primiparous and multiparous) and into other 2 groups based on their physiological status (pregnant or not). These groups were used to detect possible fluctuations in the lymphocyte subpopulations. RIs specific for each subgroup were not calculated, as they would have been poorly representative of the population, because of the low number of animals included in some groups.

A Shapiro-Wilk test was performed to assess whether the data were normally distributed. Kruskal-Wallis or ANOVA tests were performed to compare means among age- and ~~lactation~~reproductive history- groups. When a significant variation occurred, post-hoc analyses were performed with Mann-Whitney, Bonferroni or Dunnett tests, based on data distribution and homoscedasticity assessment. Mann-Whitney test was also used to compare means between pregnant and non-pregnant animals.

Significance was set at $p \leq 0.05$ for all tests except for Mann-Whitney test whereby, based on the number of possible paired contrasts, the significance threshold was set at $p \leq 0.005$ for age groups, and at $p \leq 0.017$~~08~~ for ~~lactation~~ reproductive history groups, in order to reduce the family-wise error rate (FWER) in multiple comparisons.

All tests were performed by means of a standard statistical software (SPSS v20.0 for Windows).

Results

Overall, blood samples from 112 healthy buffaloes were included in the study.

RIs for the lymphocyte~~s~~ subclasses are listed in table 1. In all samples two lymphoid populations were identifiable based on FC morphological scattergram, differing for cell size (small and medium) (Figure 1B): their respective percentages were recorded and the absolute concentrations calculated. Small and medium lymphoid cells were not clearly ~~discriminable~~ recognizable on the blood smears.

Age was <2 years in 26 (23.2%) animals, 2-4 years in 21 (18.8%), 4-6 years in 22 (19.6%), 6-8 years in 24 (21.4%), and >8 years in 19 (17.0%).~~%). DIM were recorded in 52 animals: 9 (7.3%) were <45, 5 (9.6%) between 45 and 200 days, 27 (51.9%) between 200 and 300, and 11 (21.2%) >300 days.~~ Reproductive history and pregnancy status were retrieved for 84 animals aged >2 years. Fourteen (16.7%) animals were heifers, 12 (14.3%) were primiparous and 58 (69%) were multiparous. Sixty-three (75%) animals were pregnant, and twenty-one (25%) were not.

The concentration of all lymphocyte subclasses significantly varied with age: p-value was <0.001 for all analyses. Significant results of post-hoc analyses are shown in figure 2~~1~~. Most of the significant results were obtained when comparing <2 years old animals with each other age group: the youngest animals had a higher concentration of most of the lymphocytes subclasses.

The concentration of all lymphocyte subclasses significantly varied also according to the reproductive history: p-value was <0.001 for all analyses but for the concentration of CD3+, CD4+ and CD21+ lymphocytes (p=0.024, p=0.001 and p=0.009, respectively). Significant results of post-hoc analyses are shown in figure 3. Heifers had higher concentrations of all lymphocyte subclasses, whereas only minimal differences could be detected between primi- and multiparous animals.

Finally, pregnant animals had lower concentrations of CD3+ (p<0.001; mean±sd: 1052±572 and 1665±699 cells/µl, respectively), CD4+ (p=0.050; mean±sd: 717±398 and 894±372 cells/µl, respectively) and CD8+ lymphocytes (p=0.006; mean±sd: 583±331 and 909±547 cells/µl, respectively).

~~When lactation phase was considered, significant differences among groups were obtained for the concentration of small and medium lymphocytes (p=0.049 and p=0.010, respectively) and for WC1-N2+ and WC1-N3+ lymphocytes (p=0.044 and p=0.011, respectively). However, post-hoc analyses rendered only few significant results (figure 2).~~

Different recent studies evaluated infectious diseases of water buffaloes, because of their potential influence on productivity (Roperto et al., 2013; Nogarol et al., 2014; Angeles et al., 2015; Panigrahi et al., 2016; El-Halawany et al.,2017; Hornok et al., 2018; Martins et al., 2018; Paradiso et al., 2018).

Still, ~~little is known about~~ the knowledge of water buffalo ~~the~~ immune system ~~of these animals~~ in non-pathological conditions is rather lacking. We report RIs for different lymphocyte subclasses. In addition, our results highlight a relevant difference in the concentration of the main subclasses between buffaloes < and > 2 years old and between heifers and primi- or multiparous animals.

The first considerations about biological variations and RIs date back to the 60s, with the spread of laboratory automation that allowed the collection of a large amount of data, leading to the publication of the first studies on this topic (Siest et al., 2013). ~~From then on~~Since then, many papers have been published using different methods to calculate RIs. Finally, ~~O~~official guidelines for RIs calculation and reporting have ~~finally~~ been published in the last decade both in human and veterinary medicine, aimed at tidying up this mess of different approaches (CLSI and IFCC, 2008; Friedrichs et al., 2012). Normal values and RIs have been provided in the literature for hematological and biochemical parameters in water buffaloes (Jain et al., 1982; Canfield et al., 1984; Ciaramella et al., 2005; Abd Ellah et al., 2014; Torres-Chable et al., 2017). However, to the authors' knowledge, lymphocyte subpopulations have only been analyzed in a recent study, which provided some descriptive data without calculating RIs (Grandoni et al., 2017). In this study, results are shown as percentages of CD-positive cells out of a lymphocytes-monocytes gate set on a FC morphological scattergram, which is expected to include peripheral blood mononuclear cells (PBMC). ~~Differently from~~Unlike Grandoni and colleagues, in the present manuscript we provide RIs for the absolute concentration of the main lymphocyte subpopulations in water buffaloes.

Despite the~~se~~ differences in the study design and results reporting, an effect of age on the composition of lymphocyte population was detected in both studies. Grandoni and colleagues reported a decreased proportion of γδ T-cells, CD8+ and CD21+ cells in older animals, whereas the proportion of CD4+ cells remained approximately constant with age. These differences between young and old animals are also supported by the results of the statistical analyses performed in the present study. Indeed, buffaloes <2 years old had a significant higher concentration of each lymphocyte subclass, when compared to older animals. Significant variations according to age have been reported for other

hematological and biochemical parameters in water buffaloes (Canfield et al., 1984; Ciaramella et al., 2005). The influence of age on hematological parameters was reported in ~~the~~ bovine species, as well (Lumsden et al., 1980; Brun-Hansen et al., 2006; Mohri et al., 2007; Panousis et al., 2018). Taken together, all these results support the need for age-specific RIs.

The composition of hematic lymphoid population also grossly varied with reproductive history ~~did not vary with lactation phase~~ in our study~~, except for WC1-N2+ and WC1-N3+ subpopulations. However, these results were almost not supported by the post-hoc analyses and warrant further investigations~~. Thus, it could be argued that the differences found between young and adult animals mostly rely on the nulliparous status of animals aged <2 years. Reproductive history was not considered in the study by Grandoni and colleagues (Grandoni et al., 2017), thus preventing any comparison of our results with already published literature.

Not even the pregnancy status was included in the study by Grandoni (Grandoni et al., 2017). In our cohorts of animals, only few parameters varied with pregnancy. Two recent studies from the same research group described the changes in blood chemistry during the transition period, and the metabolic and hormonal response around calving and early lactation in water buffalo cows (Fiore et al., 2017; Fiore et al., 2018), whereas no data ~~is~~ are available concerning changes in the hematological profile among different production periods in this species. ~~Conversely,~~ Interestingly, Moretti and colleagues reported a modification ~~in~~ of many hematological parameters in Holstein dairy cows at 30 DIM, when compared to the same animals sampled at 3 DIM: lymphocyte count significantly decreased between the two sampling time, but the composition of this population was not assessed by the authors (Moretti et al., 2017). Further studies are needed to define whether hematological parameters vary with pregnancy and within the ~~first month of~~ lactation period in water buffaloes, as well.

The main limitations of the present study are the relative small number of antigens tested and the application of a single-color FC approach ~~(which prevented us from assessing the contemporary expression of different antigens on the same cellular population)~~. This is of major importance for T-

cell subsets, as the different antibodies we tested recognize different epitopes of the same antigen, or different antigens co-expressed on the same cells. The T-cell population is composed of αβ and γδ subpopulations. The first one is further composed of CD4+ and CD8+ cells. However, the sum of CD4+, CD8+ and γδ T-cells does not correspond to the number of CD3+ cells, as most of γδ T-cells stain positive for CD8 in water buffaloes (Grandoni et al., 2017). Also, γδ T-cells are composed of WC1+ and WC1- cells. WC1 receptor is encoded by 13 different genes in the cattle and even more genes in other species, resulting in different isoforms (Chen et al., 2012). The antibodies we used in the present study recognize three different epitopes of WC1 receptor: WC1-N2 is expressed on all isoforms, whereas WC1-N3 and WC1-N4 are mutually expressed on different subsets of WC1-N2+ γδ T-cells in water buffaloes (Grandoni et al., 2017). The application of a single color FC approach prevented us from assessing the contemporary expression of these different antigens and epitopes on the same cellular population. Further studies are needed to amend these pitfalls.

In conclusion, this is the first study reporting the composition of the lymphoid population in the peripheral blood of a large number of healthy water buffaloes, providing specific RIs. Based on our results and in agreement with the published literature (Grandoni et al., 2017), the count of each lymphocyte subclass varies with age, being significantly higher in heifers and in animals <2 years old~~, but not with lactation phase~~. Further studies are needed to assess whether the composition of the lymphoid population varies among different management conditions.

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Table 1: Reference Intervals for lymphocyte subclasses in the peripheral blood of 112 healthy water buffaloes.

Parameter	Reference Interval (nr/µl)		Mean±standard deviation (nr/µl)	Median (nr/µl)	Min-max (nr/µl)
	Lower Limit	Upper Limit			
Small cells	838	9535	3353±2427	2270	609-11037
Medium cells	239	2725	1034±593	942	201-3234
CD3+	238	6366	1762±1530	1298	160-8673
CD4+	178	2144	915±530	830	126-2467
CD8+	162	2181	830±606	623	113-3690
CD21+	47	1982	423±493	237	35-2242
TCR-δ+	64	3507	805±1041	333	42-5828
WC1-N2+	57	3069	731±994	283	26-5103
WC1-N3+	19	826	167±188	88	8-979
WC1-N4+	30	2138	413±630	177	2-3797

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Figure 1: automated analyses of peripheral blood WBC in a healthy water buffalo. **A**: Sysmex XT 2000-iV, DIFF-channel: cells are displayed based on their light scatter (x-axys) and fluorescence signal produced by staining nucleic acids with a polymethine dye (y-axys); gates were manually created to fit with buffaline leukocyte subclasses (light blue, neutrophils; red, eosinophils; purple, lymphocytes; green, monocytes). **B**: FACScalibur: cells are displayed based on their size (x-axys) and complexity (y-axis); two different lymphoid cell populations were detected, differing for their size (green dots, small sized cell; purple dots, medium sized cells)

Figure 2~~1~~: histograms showing the mean absolute concentration of different lymphocytes subclasses in 112 healthy water buffaloes of different age. X-axis: age group (years). Y-axis: mean concentration (cells/µl). *: =significantly different from each other group at post-hoc analyses. a-f: significant difference at post-hoc analyses

Figure 3~~2~~: histograms showing the mean absolute concentration of different lymphocytes subclasses in 84~~52~~ healthy water buffaloes according to the ~~lactation phase~~reproductive history. ~~X-axis: lactation phase (1: <45 days; 2: 45-200 days; 3: 200-300 days; 4: >300 days).~~ Y-axis: mean concentration (cells/µl). *: significantly different from each other group at post-hoc analyses. a: significant difference at post-hoc analyses

~~a: significant difference at post-hoc analysis~~