Таблица 1
Иммунофенотипическое исследование лимфоцитов периферической крови, бурсы и селезёнки цыплят-бройлеров
n=3

Возраст птиц, сут.	B-лимфоциты, %	T-лимфоциты, %			
	бурса	кровь		селезёнка	
	CD45+Bu1a+	CD45+CD4+	CD45+CD8α +	CD45+CD4+	CD45+CD8α +
8	80,91±2,88	45,41±3,38	16,14±1,57	25,93±4,38	22,49±0,3
10	96,43±1,4	49,5±1,12	13,68±0,39	29,32±1,2	24,93±4,74
13	97,26±0,02	59,39±6,53	18,15±1,73	30,32±5,47	21,25±0,18
16	94,11±2,06	50,06±4,36	20,1±4,17	30,14±2,81	23,55±3,78
21	97,08±0,98	46,29±2,91	20,06±4,67	29,81±5,08	26,07±5,22

Таблица 2
Иммунофенотипическое исследование лимфоцитов периферической крови и селезёнки цыплят яичного направления
n=5

Возраст птиц, сут.	B-лимфоциты, %		T-лимфоциты, %			
	кровь	селезёнка	кровь		селезёнка	
	CD45+MHCII+		CD45+CD4+	CD45+CD8β+	CD45+CD4+	CD45+CD8β+
53	5,38±1,73	10,17±5,16	45,54±10,2	5,78±1,18	28,55±5,49	34,79±3,0
57	1,21±0,13	13,95±3,29	47,43±0,49	8,76±3,77	31,5±3,93	33,75±6,82
62	1,11±0,18	14,36±5,84	54,66±3,12	8,1±2,33	27,31±5,35	32,5±5,77
70	2,29±1,0	12,37±0,86	55,06±3,28	12,91±1,36	27,33±8,02	25,19±0,59
120	2,45±0,43	н/д	53,41±2,6	13,36±1,05	н/д	н/д

н/д — нет данных.

цитофлуориметрии с использованием прибора BD FACSVerse™. Проведенные исследования показали возможность использования разработанного метода для определения и количественной оценки различных популяций и субпопуляций лимфоцитов кур.

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UDC 619:616-074:636.52/.58

IDENTIFICATION OF T- AND B-LYMPHOCYTES IN CHICKENS AND THEIR SUBPOPULATIONS BY FLOW CYTOFLUOROMETRIC ANALYSIS USING FLOW CYTOMETER BD FACSVERSE™

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SUMMARY
Method of identification of T- and B-lymphocytes in chickens and their subpopulations by flow cytofluorometric analysis using BD FACSVerseTM was developed. Method of lymphocytic gate extraction with control of leukocyte common antigen CD45 and monocytic antigen expression was determined. Antibody panels for immunophenotyping lymphocytes when testing chicken cell mediated immune response were selected and tested. They enable to determine a relative number of T- and B-lymphocytes, T-helpers and T-cytotoxic cell populations in blood and chicken lymphoid organs. The paper demonstrates specificity and reproducibility of the developed method of identification of chicken lymphocyte subpopulation.

Key words: flow cytofluorometric analysis, immunophenotyping of chicken lymphocytes.

INTRODUCTION

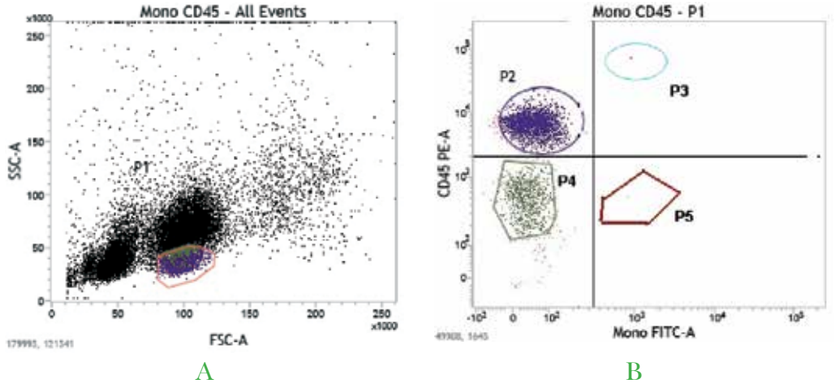
In the present time flow cytometry is increasingly applied in veterinary immunology. Usage of different sets of cell markers enables testing of different types of cells and their functional status and determination of proportion of different cell subpopulations.

Lymphocyte immunophenotyping is detection of differentiation markers or CD-antigens on their surface. CD classification (cluster of differentiation agents) is based upon differences between cell clusters in surface and differentiation markers.

Basic CD markers for assessment of cell component of chicken immunity are CD45 characterizing common pool of leucocytes and CD3-marker - mature T-lymphocytes [3, 6]. Mature T-cells (thymocytes and T-lymphocytes) express T-cell receptor (TCR) together with CD3 on cell membrane including different chains of T-cell receptor TCR TCRαβ and TCRγδ (TCR1) [8]. Together they form a so called TCR-CD3-complex. Other T-cell markers (CD4, CD8α, CD8β) make it possible to characterize immunophenotype of T-cells during further differentiation stages. CD4 express thymocytes, T-lymphocytes (T-helpers) and monocytes/macrophages [4]. CD8α and CD8β recognize thymocytes, cytotoxic T- lymphocytes of two different subpopulations of T-cells (homo α/α- and getero α/β-dimers). Homodimers are detected mostly in young birds [5, 7].

Bu1a and Bu1b are expressed on the surface of cell membrane of B-lymphocytes mostly of corresponding inbrend chickens lines (Bu1a и Bu1b). Bu1 antigen recognizes B-lymphocytes of both chicken lines. Bu1, Bu1a, Bu1b are also expressed on the surface of macrophages and monocytes but not erythrocytes, granulocyte and thrombocyte. MHC-II antigen (major histocompatibility complex, II class) recognizes B-lymphocytes and antigen presenting cells [5, 6].

Flow cytometry is based on registration of light scattering and fluorescence of cells in suspension, one after another passing through homogeneous beam of light with the stream (usually laser light). Photometric channels are used for assessment of cell size and cell structure. Fluorescent channel is used for studying cell markers. For this purpose fluorochrome labeled monoclonal antibodies to membrane and intracellular components (proteins, DNA and RNA) are used [1]. Development of a new modern multicolor flow cytometry technique and computer based methods of data processing expanded opportunities of this method. The investigation was aimed at devel-



A B

Fig. 1. Immunophenotypic tests of peripheral blood cells
A – lymphocyte gate extraction (P1) in forward scattering and (FSC) side scattering (SSC) channels;
B – dot diagram of cell distribution into two populations (monocytes and leucocytes): P2 (Mono-/CD45+) – leucocytes, P3 (Mono+/CD45+) – monocytes; P4 (Mono-/CD45-) and P5 (Mono+/CD45-) – debris.

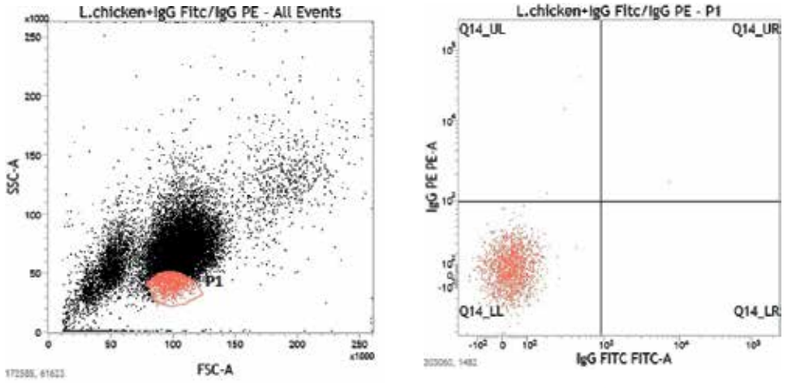
oping a method of identification of T- and B-lymphocytes in chickens and their subpopulations by flow cytofluorometric analysis using BD FACSVerse™.

MATERIALS AND METHODS

Lymphocyte extraction from chicken lymphoid organs and blood. For extraction of lymphocytes from chicken lymphoid organs (spleen, bursa) organs collected in conformance with aseptic rules were immerged in buffer solution (0,1M phosphate buffer with 0,85% NaCl, pH 7,2). Then a suspension was prepared using cells by putting organs through a sterile sieve into a necessary RPMI 1640 medium volume (1:10 of sample weight). Cell suspension was layered onto Ficoll-Paque™ gradient (Amersham Biosciences, Sweden) (1,007 density) in 2:3 proportion and centrifuged at 453 g for 30 min. Extracted lymphocyte faction was washed with buffer solution 1 time by centrifuging at 300 g for 10 min. Cell pellet was resuspended in 1 ml of RPMI-1650 medium. Lymphocyte extraction from chicken peripheral blood was carried out according to the standard procedure [1] using Ficoll-Paque™ PLUS. Cell count and determination of their viability was carried out using Countess Automated Cell Counter (Invitrogen, Korea). Lymphocyte concentration was adjusted to 10^6–10^7cell/ml using buffer solution.

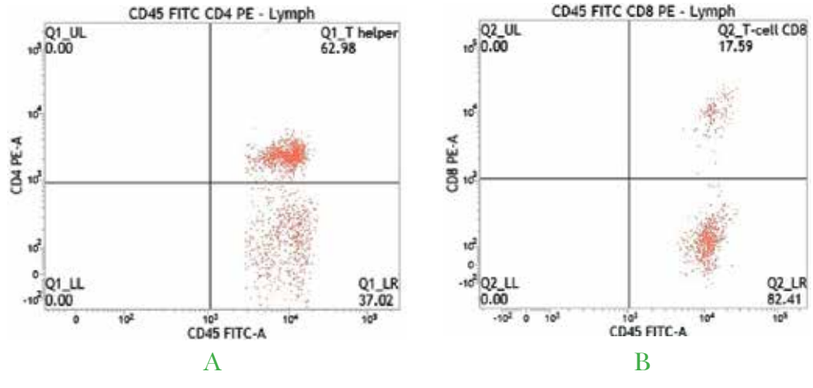
Sample preparation for detection of lymphocyte surface markers. Monoclonal antibodies conjugated to fluorochrome (Fluorescein isothiocyanate – FITC, phycoerythrin – PE) in a recommended by the manufacturer volume (Southern Biotech, США; Serotec, Англия) were added to 50 mcl of prepared samples in several replicates (depending on a number of used antibody panels). The samples were incubated for 30 min at 4–8°C. They were washed from unbounded antibodies by centrifuging for 10 min at 260 g in buffer solution. The pellet was resuspended in 1,0 ml of stream for measuring using the cytometer.

Quantitative analysis of lymphocyte subpopulations. Analysis of immunophenotypic characteristics of cells was carried out using flow cytometer BD FACSVerse™ (BD Bioscience, США). After calibration of the device using BD FACSuite™ FC Beads-4c Research kit and BD FACSuite™-Research Beads (USA) tested samples were measured according to operational guidelines and measured cell



A B

Fig. 2. Immunophenotypic tests of peripheral blood cells
A – lymphocyte gate extraction P1 in forward scattering and side scattering channels;
B – determination of quadrant boundaries for fluorescence detectors (FITC u PE) in conformance with the size of the isotopic control gate.



A B

Fig. 3. Determination of basic T-lymphocyte subpopulations in chicken peripheral blood using monoclonal antibody combinations CD45FITC/CD4PE and CD45FITC/CD8PE
A – dot diagram showing CD45+CD4+ lymphocytes (T-helpers) in the right upper quadrant;
B – dot diagram showing CD45+CD8+ lymphocytes (T-cytotoxic lymphocytes) in the right upper quadrant.

populations were analyzed using BD FACSuite Software: Worklist Workflow or Expirement Workflow.

Experimental animals. 8–21 day old commercial broilers (15 birds) and 53–120 day old egg laying chickens (25 birds) were used for blood sampling.

Statistical result analysis. For statistic data analysis Statistica 6.0 was used.

RESULTS AND DISCUSSION

Development of a method of identification of T- and B-lymphocytes in chickens and their subpopulations by flow cytofluorometric analysis using BD FACSVerse™ several stages.

At the first stage it was necessary to determine the method of extracting lymphocyte gate of the cell subpopulation. According to publications there are several methods of lymphocyte gate extraction [1, 2]. Method of lymphocytic gate extraction with control of leukocyte common antigen CD45 and monocytic antigen expression was chosen by means of experiments (Fig. 1).

Maximum number of events are shown in upper left quadrant (gate P2) of diagram CD45 PE/CD monocyte/macrophage FITC.

The next stage was to select isotopic control for determination of markers of fluorescence intensity and nonspecific staining. IgG FITC/IgG PE, containing labeled FITC и PE anti-mouse immunoglobulins G (IgG) (Fig. 2), was selected as an effective isotopic control.

According to publications antibody panels for lymphocyte immunophenotyping were designed and tested during studies of chicken cellular immune response:

- CD45FITC/CD3PE –T-lymphocytes;
- CD45FITC/CD4PE – T-helpers;
- CD45FITC/CD8PE – citotoxic T-lymphocytes;
- TCRαβFITC /TCRγδPE – T-lymphocytes;
- CD45FITC/MHC-IIPE – B-lymphocytes, antigen-presenting cells;
- CD45FITC/Bu1PE – B-lymphocytes.

Fig. 3 demonstrates results of determination of basic T-lymphocyte subpopulations (CD4 and CD8) in chicken peripheral blood using selected antibody panels.

Specificity of the method was assessed by immunophenotyping of chicken peripheral blood lymphocytes conjugated to monoclonal antibodies to human, bovine, porcine and chicken cell surface markers (Fig. 4)

Positive events on dot diagram for PE/FITC were reported only for chicken lymphocytes conjugated to chicken CD45 and CD3 monoclonal antibodies.

Intra-laboratory reproducibility was assessed by immunophenotyping of chicken peripheral blood lymphocytes conjugated to chicken CD45, CD3, CD4 and CD8β monoclonal antibodies. The test was performed in four replicates. Test results are shown in Fig. 5

Coefficient of variation of the results for each cell population was not over 3%; difference between minimal and maximal values at 95% confidence level did not exceed 2% thus indicating reproducibility of the method [1, 2].

Subpopulations of immunocompetent cells in chicken peripheral blood and lymphoid organs were tested using the developed method. The results are shown in Tables 1–2.

The data demonstrate that percentage of basic populations of immunocompetent cells in broiler chicks included: T-helpers – 42–66% in blood and 25–35% in spleen; cytotoxic T-cells – 14–25% in blood and 20–31% in spleen. B-lymphocytes in broiler chicken bursa amounted to 78–99% (Table 1).

In egg laying chickens T-helpers amounted to 35–58% in blood and 19–35% in spleen. Percentage of cytotoxic T-cells amounted to 4,5–14% in blood and 25,5–40,5% in spleen. B-lymphocytes amounted to 5–20% in spleen and 1–7% in blood (Table 2).

The obtained results were consistent with percentage of the studied immunocompetent cell populations in chicken peripheral blood and lymphoid organs reported by the manufacturers of monoclonal antibodies intended for use in flow cytometry.

CONCLUSION

Therefore, specific and reproducible method for immunophenotyping of chicken blood and lymphoid organ cells by flow cytometry using BD FACSVerse™ was developed. The studies demonstrated that the developed method could be used for detection and assessment of different chicken lymphocyte populations and subpopulations.

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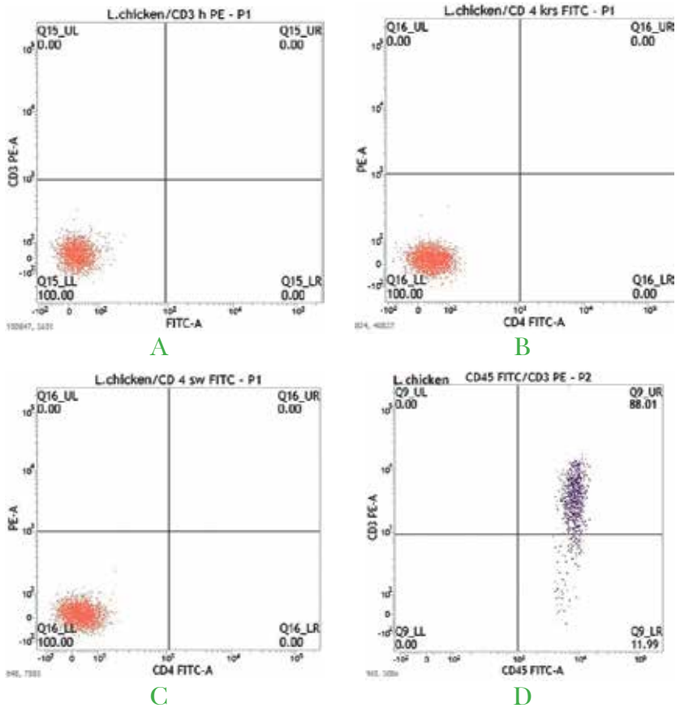

Fig. 4. Immunophenotyping of chicken peripheral blood cells
A – dot diagram for chicken lymphocytes/ human CD3PE; 100% of CD3PE-/FITC- events are shown in left lower quadrant;
B – dot diagram for chicken lymphocytes/ bovine CD4FITC; 100% of PE-/CD4FITC- events are shown in left lower quadrant;
C – dot diagram for chicken lymphocytes/ porcine CD4FITC; 100% of PE-/CD4FITC- are shown in left lower quadrant;
D – dot diagram for chicken lymphocytes/ chicken CD45FITC/CD3PE; 88.01% of CD45+FITC/CD3+PE cells (T-lymphocytes) are shown in right upper quadrant

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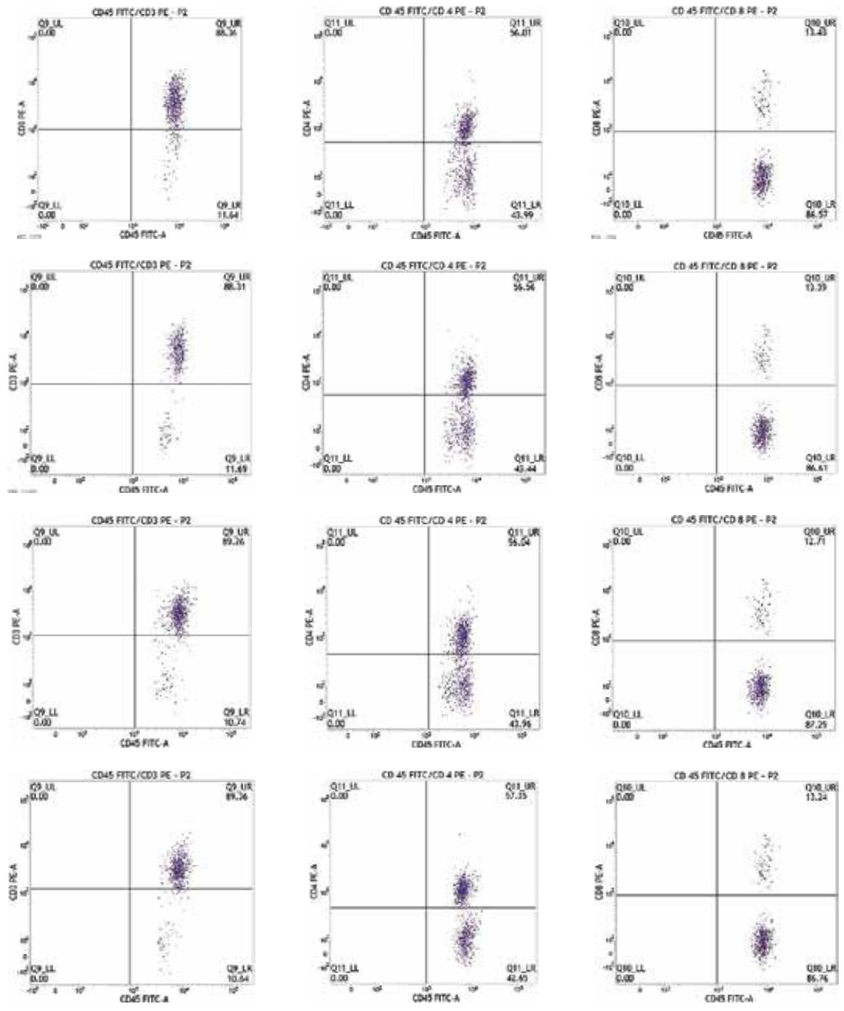
8. T lymphocyte subpopulations diverge in commercially raised chickens / B.W. Bridle, R. Julian, P.E. Shewen [et al.] // Can. J. Vet. Res. – 2006. – Vol. 70. – P. 183–190.

Table 1
Immunophenotyping of lymphocytes of chicken broiler peripheral blood, bursa and spleen
n=3

Age of birds, days	B-lymphocytes, %	T-lymphocytes, %			
	bursa	blood		spleen	
	CD45+Bu1a+	CD45+CD4+	CD45+CD8α +	CD45+CD4+	CD45+CD8α +
8	80,91±2,88	45,41±3,38	16,14±1,57	25,93±4,38	22,49±0,3
10	96,43±1,4	49,5±1,12	13,68±0,39	29,32±1,2	24,93±4,74
13	97,26±0,02	59,39±6,53	18,15±1,73	30,32±5,47	21,25±0,18
16	94,11±2,06	50,06±4,36	20,1±4,17	30,14±2,81	23,55±3,78
21	97,08±0,98	46,29±2,91	20,06±4,67	29,81±5,08	26,07±5,22



88,82±0,28[1]
87,92÷89,72[2]
0,64[3]
A

56,49±0,31
55,49÷57,49
1,11
B

13,19±0,17
12,66÷13,72
2,5
C

Fig.5 Immunophenotyping of chicken peripheral blood cells
A – dot diagram for CD45FITC/CD3PE; CD45+CD3+ lymphocytes (T-lymphocytes) are shown in right lower quadrant
B – dot diagram for CD45FITC/CD4PE; CD45+CD4+ lymphocytes (T-helpers) are shown in right lower quadrant
C – dot diagram for CD45FITC/CD8βPE; CD45+CD8+ lymphocytes (cytotoxic T-cells) are shown in right lower quadrant;
[1] mean value ± standard error of mean; [2] 95% confidence level; [3] coefficient of variation.

Table 2
Immunophenotyping of lymphocytes of egg laying chicken peripheral blood and spleen
n=5

Age of birds, days.	B-lymphocytes, %		T-lymphocytes, %			
	blood	spleen	blood		spleen	
	CD45+MHCII+		CD45+CD4+	CD45+CD8β+	CD45+CD4+	CD45+CD8β+
53	5,38±1,73	10,17±5,16	45,54±10,2	5,78±1,18	28,55±5,49	34,79±3,0
57	1,21±0,13	13,95±3,29	47,43±0,49	8,76±3,77	31,5±3,93	33,75±6,82
62	1,11±0,18	14,36±5,84	54,66±3,12	8,1±2,33	27,31±5,35	32,5±5,77
70	2,29±1,0	12,37±0,86	55,06±3,28	12,91±1,36	27,33±8,02	25,19±0,59
120	2,45±0,43	n/d	53,41±2,6	13,36±1,05	n/d	n/d