QUALITATIVE AND QUANTITATIVE PARAMETERS OF SWINE CELL IMMUNITY

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Flow cytometry is a modern analytic method for qualitative and quantitive analyses of procaryotic and eukaryotic cells. Classical analytical methods examine one characteristic as an average for an examined cell population. Flow cytometry simultaneously measures several biochemical and physical parameters of every single cell in a heterogenic cell sample. Flow cytometry is routinely used all over the world in professional laboratories in veterinary medicine. For that purpose on the Veterinary Faculty of University in Zagreb, a machine for flow cytometry was standardized for use in veterinary medicine, especially for immunophenotyping of haemopoetic cells of domestic animals. In this manuscript we showed the standardisation method on a swine model, on samples of peripheral blood for the immunophenotypification of $CD45^+$, $CD3^+$ and $CD21^+$ cells.

Key words: flow citometry, immunophenotypization, swine

INTRODUCTION

Flow cytometry is a contemporary analytical method for qualitative and quantitative analysis of prokaryotic and eukaryotic cells (Šiftar and Kardum Paro, 2003). What makes it different from classic analytical methods which measure one parameter as an average value of the entire cell population is that it can simultaneously measure several physical and biochemical parameters of every single cell in the heterogeneous cell sample (Ormerod, 2000).

Researchers have shown a great interest for investigating the swine immune system, with emphasis on the similarities of its structure and function to the human immune system (Binns and Pabst, 1996). By reviewing scientific and professional literature it is clear that the method of flow cytometry is becoming important in the evaluation of swine immune status through the distribution of different leukocyte immunophenotypes of healthy pigs during the production period (Dorn et al. 2002).

Regarding the fact that the use of flow cytometry in veterinary medicine in Croatia is still not sufficient and that the Department of Biology at the Veterinary

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Faculty in Zagreb possesses a device for flow cytometry Epics XL (Beckman, Coulter, USA), the aims of this investigation were:

− to establish the importance of cytometry by determining standard profiles of immune cell elements of clinically healthy pigs regarding sex;

− to establish the potential for applying flow cytometry as a useful method in diagnostics, prevention and therapy assessment of sick animals in the field and clinical laboratories.

MATERIALS AND METHODS

Biological material: The investigation was conducted on 10 clinically healthy pigs (Swedish Landrace X Yorkshire), of both sexes, at the age of 3 months, with an average weight of 30 kg, born and raised on a farm in northwestern Croatia. The pigs were divided in two groups according to sex: group A (5 male animals); group B (5 female animals). During the trial the animals were kept in rearing pens and were fed with feedstuff for growing-finishing pigs *ad libitum*.

Animals were treated according to the European document for keeping and handling laboratory animals: Directive for the Protection of Vertebrate Animals Used for Experimental and Other Purposes (86/609/EEC).

Samping: A 2 mL of peripheral blood sample was taken by veinpunction from the jugular vein of each pig and placed into tubes containing anticoagulant (Beeton Dickinson vacutainer). Samples were stored at +4ºC.

Monoclonal antibodies and conjugates: The monoclonal antibodies (mAbs) specific for swine leucocyte differentiating antigens (CD receptors) were kindly donated by dr. Karin Haverson, prof. dr. sc. Christofera C. Stokes (Division of Molecular and Cellular Immunology, School of Veterinary Science, University of Bristol, Bristol, United Kingdom), and dr. A. Saalmüller (Federal Institute for Viral Diseases of Animals, Tübengen, Germany) (Table 1).

Table 1. Characteristics of monoclonal antibodies used for investigations of swine leucocyte subpopulations in peripheral blood by flow cytometry

CD antigen	Isotype	Marker	Cells	Donor*
CD45+	IgG1	MIL5	Leukocyte	Pescovitz
CD3+	IgG1	BB23-8E6	T-lymphocyte	Haverson
CD21+	IgG1	BB6-11c9	B-lymphocyte	Pescovitz

*Head of the laboratory where monoclonal antibodies for 2nd (Davis, CA, USA, 1995) and 3rd Swine CD Workshop (Ludhiana, India, 1998. and Amsterdam, Holland, 1999) were produced

As secondary antibody we used rabbit against mouse IgG conjugated with flourescine-isothiocynate (FITC) (Department of Immunology, Zagreb)

Hematological tests: By using the automatic hematological counter (ADVIA 120 Hematology System, Bayer Diagnostics, Germany) 10 samples of swine peripheral blood (1 mL) were analyzed. The differential blood counts from blood

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smears were determined, as well. The specific subpopulations of leucocytes in peripheral blood were determined by examining the blood smears after coloring them by Giemsa.

Flow cytometry: The numbers of leukocytes in peripheral blood samples (100 μL) were analyzed by the flow cytometer (Coulter EPICS-XL). Blood samples were previously diluted with PBS buffer until the leukocyte numbers were between 5.0 - 9.7 x 10^9/L. We added 50 μL of swine CD^+ lymphoid marker monoclonal antibodies to the 100 μL of diluted blood, incubated for 20 minutes at room temperature, and washed with 2 mL of buffered phosphate solution (PBS) by centrifuging for 5 minutes at 2000 rpm. After centrifuging the supernatant was removed, and 50 μL of FITC marked secondary antibodies (rabbit anti mouse IgG) to the pellet were added and incubated for 20 minutes at room temperature. Samples were washed by centrifuging for 5 minutes at 2000 rpm with 2 mL of PBS, and the pellets were treated with 2 mL of lytic reagent (ammonium chloride, NH_4Cl, pH 7.3) and left in the dark, at room temperature for 10 minutes. Samples were triplicated and each was analyzed for 10 000 cells on the flow cytometer.

Statistical data analysis: The results were analyzed by Mann-Whitney test and t-test. During statistical analysis ANOVA software was used.

RESULTS

Hematological analysis results: In this paper the hematological analysis of the acquired data on leukocyte and erythrocyte count, and the percentage of segmented and unsegmented cells, eosinophiles, lymphocytes and monocytes in the overall leukocyte population did not yield a statistically significant difference (p>0.05) with regard to swine sex (group A - male; group B - female) (Table 2).

Table 2. Hematological values of swine peripheral blood in Group A and Group B

	Er (x 10^{12}/L)	Le (x 10^9/L)	PMN se (%)	PMN nse (%)	Eo (%)	Ly (%)	Mo (%)
Group A (N=5)	6.1±0.32	21.4±2.1	54.8±3.4	3.6±5.2	1±2.7	37.3±3.2	2.9±2.5
Group B (N=5)	6.3±0.27	22.1±3.5	52.9±2.8	2.9±4.8	0.5±3.9	41.8±5.2	1.9±3.3

Group A (5 male animals); Group B (5 female animals); se - segmented, nse - nonsegmented

Results of single color flow cytometry: Results acquired by the method of single color flow cytometry in this paper are presented in the form of a two-parameter histiogram. On the basis of laser beam dissemination, proportional to cell size (forward light scatter-FS), and the degree of light dissemination at 90° from the direction of the beam, proportional to cell segmentation (side scatter - SC) the data presented (Figure 1) shows cell size and cytoplasm segmentation of swine leucocytes subpopulations: lymphocytes, granulocytes and monocytes.

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On the histiogram there are three separated gates (A, B, C) inside which are leukocyte subpopulations depending on cell size and cytoplasm segmentation. Cells inside gate A are the smallest in size and least segmented and their physical characteristics correspond to the lymphocyte type of cells. Cells of greater size and segmentation, the monocytes, are inside gate B. Granulocyte type cells are inside gate C. They are the largest and the most segmented cells (Figure 1).

The use of monoclonal antibodies as specific markers of swine leukocyte populations CD45$^+$ visualized (Figure 2) and determined the percentage of lymphocytes, granulocytes and monocytes from 10 000 analyzed cells by single color flow cytometry in every studied blood sample (Table 3). On the basis of acquired results there were no significant statistical differences ($p > 0.05$) regarding sex (group A – male; group B – female).

By using of monoclonal antibodies for specific CD3$^+$ swine T-lymphocyte markers from the lymphocyte gate (Figure 2) are visualized (Figure 3) and a
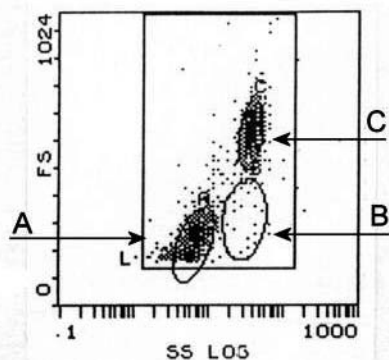

Figure 1. Swine peripheral blood leukocyte cytometric separation display depending on their size and segmentation. Histiogram displays three gates (A = lymphocytes; B = monocytes; C = granulocytes), which envelop leukocyte subpopulations
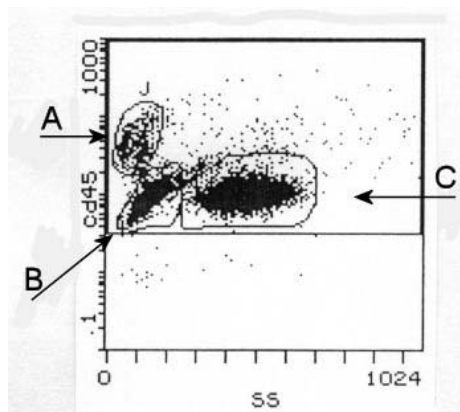
Figure 2. Swine peripheral blood leucocytes cytometric separation display depending on the presence of CD45$^+$ markers on their surface. Histiogram shows three gates (A = lymphocytes; B = monocytes; C = granulocytes), which encircle leukocyte subpopulations

percentage of T-lymphocytes is determined in relation to 10 000 analyzed cells in every sample of full swine venous blood in the trial (Table 4). There was no significant difference ($p > 0.05$) regarding swine sex (group A – male; group B – female) on the basis of the acquired data.

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Table 3. Lymphocyte, monocyte and granulocyte percentages of a heterogeneous sample of full swine blood depending on the presence of CD45$^+$ markers on their surface by single color flow cytometry

	Leucocyte subpopulations		
	Lymphocytes (%)	Monocytes (%)	Granulocytes (%)
Group A (N=5)	35.4 ± 2.3	3.6 ± 5.4	60.9 ± 6.1
Group B (N=5)	40.1 ± 6.7	2.7 ± 3.2	57.3 ± 5.9

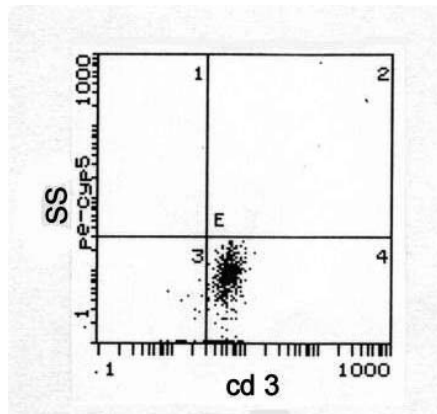Group A (5 male animals); Group B (5 female animals)

Figure 3. Cytometric display of swine peripheral blood T-lymphocyte separation depending on the presence of CD3$^+$ markers on their surface
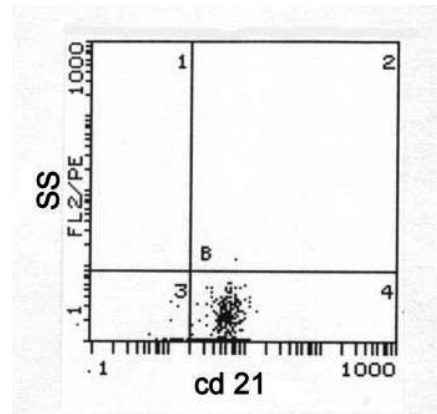

Figure 4. Cytometric display of swine peripheral blood B-lymphocyte separation depending on the presence of CD21$^+$ markers on their surface

Table 4. Percentage of T-lymphocytes in a heterogenic sample of full venous swine blood depending on the presence of surface CD3$^+$ markers with the use of single color flow cytometry

Piglet groups in trial	T-lymphocytes (%)
Group A (N=5)	51.3 ± 5.1
Group B (N=5)	48.8 ± 2.5

Group A (5 male animals); Group B (5 female animals)

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By applying monoclonal antibodies for specific CD21$^+$, swine B-lymphocyte markers from the lymphocyte gate (Figure 2) are visualized (Figure 4) and a percentage of B-lymphocytes is determined in relation to 10 000 analyzed cells in every sample of full swine venous blood in the trial (Table 5). There was no significant statistical difference ($p > 0.05$) regarding swine sex (group A – male; group B – female) on the basis of acquired data.

Table 5. Percentage of B-lymphocytes in a heterogenic sample of full venous swine blood regarding the presence of surface CD21$^+$ markers with single color flow cytometry

Piglet groups in trial	B-lymphocytes (%)
Group A (N=5)	43.7 ± 4.7
Group B (N=5)	46.2 ± 3.2

Group A (5 male animals); Group B (5 female animals)

DISCUSION

Flow cytometry is a contemporary analytical method for qualitative and quantitative assessment of biological and physical characteristics of prokaryotic and eukaryotic cells (Longobardi Givan, 1992). Today in veterinary medicine, flow cytometry is routinely applied in clinical laboratories for the assessment of the immune status of healthy animals during the production cycle (Quade and Roth, 1999). However, the application of this method is important in veterinary medicine and in the fields such as hematology, immunology, transplant medicine, oncology, genetics, toxicology and transfusion medicine (Popović and Valpotić, 2004). Furthermore, by reviewing literature data we can see that this method is useful important in veterinary medicine for the evaluation of the means and measures of prevention, diagnosis, and therapy and in monitoring the recovery of sick swine in the fields of veterinary immunology (Terzić *et al*., 2002). During flow cytometry the assessment of immunophenotype, as well as the application of specific monoclonal antibodies for differentiating leukocyte antigens – markers (membrane and/or intracellular glycoproteines) provides the data for relative proportions of certain cell subsets from the entire leukocyte population (Zuckermann and Husmann, 1996).

Investigation of surface antigens from swine leucocytes by flow cytometry provides data about leukocyte subpopulations and their role in specific immune reactions (Pescovitz *et al*., 1998a). Literature data, as well as data acquired in this trial, suggests that all swine leucocytes, present on their surface large glycolysed molecules called CD45 (or antigens common for leucocytes) (Pescovitz *et al*., 1994). It is obvious that widely dispersed proteins are in fact transmemrane receptors of tyrosine-phosphate activity. Monoclonal antibodies directed against

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these molecules, called CD45RA, CD45RB and CD45RC, differentiate the subtypes of T-cells (Zella *et al*., 1998).

The most reliable markers on peripheral T-lymphocytes are CD3 molecules, and are bound to T-cell receptors (TCR) and except for stabilizing the antigenic receptor they have a role in the transmission of the activation signal into the cell. Considering that the CD3 molecular domain is combined with TCR, these molecules represent the most reliable marker for swine peripheral T-lymphocytes (Pescovitz *et al*., 1998b). In this study flow cytometry was used to determine the proportion of T-lymphocytes in adult swine, from the entire leukocyte population of peripheral blood. Results regarding differences between sexes correspond to literature data i.e. 48.8% (female) and 51.3% (male).

CD4 and CD8 molecules recognize genetic products of the main histocompatibility complex (MHC). $CD4^+$ lymphocytes recognize the molecules from class I of the MHC system and $CD8^+$ lymphocytes recognize molecules from class II of the MHC system (Tizzard, 2002). The main difference from other animal species is that swine have two subpopulations of $CD8^+$ T-cells from which one expresses much larger $CD8^+$ antigen density than the other. The $CD8^+$ T-cell subpopulation that expresses the CD8 molecule with the greatest density includes classic cytolitic T-lymphocytes that recognize the effector cell in connection with class I MHC molecules. Functions of T-cells that express small $CD8^+$ antigen density are NK activity *in vitro* (Sallmuller *et al*., 1994) and helper activity (Pescovitz *et al*., 1994). T-cell subpopulation, known as double positive cells ($CD4^+$ $CD8^+$), is a characteristic of the swine immune system (Zuckermann and Husmann, 1996). A quantitatively significant subpopulation of double negative ($CD4^-$ $CD8^-$) T-cells is present in the circulating swine lymphocytes (Sallmuller *et al*., 1987).

The most important differentiating marker of B-lymphocytes is an immunoglobuline molecule that is also an antigen specific receptor. On the surface of B-cells differentiating CD^+ antigens are present. Two of which are specific for swine B-lymphocytes; molecule of IgM and $CD1^+$ which is present in 50% of mature swine lymphocytes. The CD21 molecule is a marker that is found only on activated B-lymphocytes (Terzić *et al*., 2002). In this study the use of single color flow cytometry in adult swine established that the portion of B-lymphocytes in the peripheral blood lymphocyte population regarding sex from 43.7% (male) to 46.3% (female) which corresponds to the available literature data.

Implementation of flow cytometry is justified in the assessment of the immune status of animals affected by economically important diseases that cause up to 50% mortality among young pigs in intensive production. Losses are further increased by reduced performance. For example in swine such diseases are classic swine fever and colidiarrea. Prophylactic measures for the eradication of such diseases are based on nonspecific protection and immunoprophylaxis and the results are easily monitored and analyzed by flow cytometry. For example, implementation of flow cytometry with monoclonal antibodies specific for swine leucocytes can monitor the humoral and cellular immunity after the application of specific immunogenic strains K or gpE2 of the classic swine fever virus envelope with or without a modificator of the immune response in 85 day old pigs from litters

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vaccinated against classic swine fever (Terzić *et al*., 2002). However, immunodeficiency was first recorded in piglets presenting low proportions of $CD4^+$, $CD8^+$, $CD21^+$, $CD45^+$. Application of a specific vaccine in the same piglets during a period of 28 days caused a change in the percentage of certain lymphocyte subpopulations that were observed, monitored and visualized by the use of flow cytometry. Also, with the use of flow cytometry, nonspecific immunomodulating effects of levamisole after immunization of weaned piglets with non-ETEC strains $F4ac^+$ or $F18^+$ was investigated. On the basis of flow cytometry analysis with monoclonal antibodies to swine lymphocytes, the activation of $CD3^+$ and $CD6^+$ T-lymphocytes, $CD45^+$ memory cells, but not the $SWC7^+$ B-lymphocytes, was established (Božić *et al*., 2003).

By using flow cytometry to evaluate $CD2^+$, $CD4^+$ and $CD8^+$ in peripheral blood lymphocyte subpopulations in pigs orally infected with *Lactobacillus salivarius.* and *E. coli*, a stronger immune response to *Lactobacillus salivarius* in relation to *E. coli* was determined (Revajova *et al*., 2000).

Further development of flow cytometry is relying on the discovery of new specific tests as markers of many still uninvestigated cell functions, specially cell metabolism disorders, and on these grounds will become an unavoidable method for the diagnosis of many diseases in veterinary medicine. In this study, during the use of flow cytometry it is evident that this method is objective and reproducibile, and as such is useful to determine the immune status of healthy pigs of both sexes and different age groups. Moreover, cytometry is important as it can provide standard profiles of immune cell elements for different animal species. High sensibility of the flow cytometer and immunophenotypization with monoclonal antibodies provide great possibilities in determining cellular immunity (in peripheral blood, and in solid lymphatic tissues) of sick animals. Thus, it provides essential data for evaluating the strength, direction and result of the immune response to antigens, as well as for therapy in specific cases and prevention in cases of exposure to pathogens.

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KVALITATIVNE I KVANTITATIVNE KARAKTERISTIKE CELULARNOG IMUNITETA SVINJA

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SADRŽAJ

Protočna citometrija (eng. flow cytometry) je suvremena analitička metoda za kvalitativnu i kvantitativnu analizu prokariotskih i eukariotskih stanica. Za razliku od klasičnih analitičkih metoda koje ispituju jedno svojstvo kao prosjek cjelokupne ispitivane stanične populacije, protočna citometrija istovremeno mjeri nekoliko biokemijskih i fizičkih parametara svake pojedinačne stanice unutar heterogenog uzorka stanica. Protočna citometrija rutinski se u svijetu primjenjuje u stručnom radu kemijskih laboratorija u veterinarskoj medicini. U tu svrhu na Veterinarskom fakultetu Sveučilišta u Zagrebu, aparat za protočnu citometriju standardiziran je za primjenu u veterinarskoj medicini i to prvenstveno za imunofenotipizaciju hematopoetskih stanica. U ovom radu prikazana je standardizacija metode na modelu svinjskog sustava na uzorcima periferne krvi i to za imunofenotipizaciju CD45$^+$, CD3$^+$ i CD21$^+$ stanica.