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Flow cytometry sorting of cells infected with African swine fever virus

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SUMMARY

The African swine fever panzootic is continuing to spread, and the number of affected countries and material losses are increasing. In particular, India, Papua New Guinea, Malaysia, Greece and Bhutan joined the list of ASF infected countries in 2020–2021. The disease control is hindered by the lack of commercially available and effective vaccines, which, in its turn, is attributable to the insufficient knowledge of ASF pathogenesis and immune defense against the disease. The use of attenuated virus variants enables a thorough investigation of the factors influencing the virulence of African swine fever virus and the immune response to it. This involves the use of naturally attenuated virus variants, as well as of the variants attenuated by a long-term passaging of the virus in cell cultures. However, virulence heterogeneity characteristic of the ASF virus population, necessitates the additional selection of infected cells for the virus cloning. Conventional culture-based techniques for virus particle cloning are rather time- and labour-consuming; it is therefore appropriate to use flow cytometry cell sorting for the selection and cloning of virus infected cells with a view of selecting homologous virus lineages. The paper presents the results of sorting of African green monkey kidney cells (CV-1) and porcine bone marrow cells infected with African swine fever virus; the cells were sorted into the 96-well culture plates using a MoFlo Astrios EQ cell sorter in order to isolate a population of the virus originating from one infected cell. After the single cell sorting of the infected cell cultures into the 96-well plates, ASF positive cell detection rates in the plate wells were 30% for porcine bone marrow cells and 20% for CV-1.

Keywords: African swine fever, cytometry, cell sorting, cell culture

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Сортировка клеток, инфицированных вирусом африканской чумы свиней, методом проточной цитометрии

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РЕЗЮМЕ

Панзоотия африканской чумы свиней продолжает свое распространение, а число пораженных стран и материальные потери увеличиваются. Так, в 2020–2021 гг. к перечню неблагополучных по африканской чуме свиней стран добавились Индия, Папуа – Новая Гвинея, Малайзия, Греция и Бутан. Борьбу с заболеванием затрудняет отсутствие коммерчески доступных и эффективных вакцин, что, в свою очередь, обуславливается недостатком знаний о патогенезе и иммунной защите при африканской чуме свиней. Детальное изучение факторов, влияющих на вирулентность вируса африканской чумы свиней и вызываемого им иммунного ответа, становится возможным при использовании его аттенуированных вариантов. Для этого применяют как естественно аттенуированные варианты вируса, так и варианты, аттенуированные в ходе длительного пассирования вируса на культурах клеток. Однако гетерогенность по признаку вирулентности, свойственная популяции вируса африканской чумы свиней, требует проведения дополнительного отбора инфицированных клеток с целью клонирования вируса. Классические культуральные методы клонирования вирусных частиц достаточно длительны и трудоемки, поэтому для отбора и клонирования инфицированных вирусом клеток с целью получения гомологичных вирусных линий целесообразно использовать сортировку клеток методом проточной цитометрии. В данной работе показаны результаты сортировки зараженных вирусом африканской чумы свиней клеток почки африканской зеленой мартышки CV-1 и костного мозга свиньи с помощью клеточного сортера MoFlo Astrios EQ в 96-луночные культуральные планшеты с целью получения популяции вируса, происходящего из одной зараженной клетки. После проведения сортировки инфицированных культур клеток по одной клетке в лунки 96-луночных планшетов частота обнаружения положительных на африканскую чуму свиней клеток в лунках составила 30% для клеток костного мозга свиньи и 20% – для CV-1.

Ключевые слова: африканская чума свиней, цитометрия, сортировка клеток, культура клеток

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INTRODUCTION

African swine fever (ASF) is a contagious septic disease that affects both domestic pigs (including miniature ones) and wild boar. Susceptible animals can develop an acute, subacute, chronic or asymptomatic form of the disease [1, 2]. The nature of the disease manifestations depends on the biological properties of the causative virus, as well as on the individual characteristics of an animal's immune system structure. Global ASF situation continues to deteriorate and threaten the world's food security. Currently, over 50 countries are affected by the disease, and more countries are constantly getting added to the list. In 2019, African swine fever was reported in Mongolia, Vietnam, Cambodia, North Korea, South Korea, Laos, Slovakia, Serbia, Myanmar, Indonesia, the Philippines and Timor. Greece, Papua New Guinea, India and Germany joined the ranks of ASF infected countries in 2020, Malaysia and Bhutan – in 2021. Thus, the disease continues to spread and affect the countries located on various continents and differing in population size, agriculture and veterinary legislation development levels. ASF-associated losses increase with the growing number of the countries affected with the disease. In particular, the ongoing epizootic in China, with more than 100 million pigs destroyed and dead, has had a serious impact on the global pig production sector [3–5].

Despite the recent progress achieved in the studies of the ASF agent and the development of specific ASF prophylaxis means, there are still no commercially available and effective vaccines. One of the reasons for this is the insufficient knowledge of ASF pathogenesis and immune defence against the disease. At the same time, ASF virus itself possesses a number of mechanisms to escape the carrier's immune response, which include the ability to effectively replicate in macrophages, to alter cytokine and interleukin production, as well as to escape neutralization by specific antibodies [6, 7].

Studying these processes involves the use of ASF virus strains that differ in virulence, contagiousness, reactogenicity, the severity of induced clinical signs and include both naturally attenuated strains and those attenuated by a long-term passaging of the virus in cell cultures. Such strains are necessary for the identification of ASFV virulence and pathogenicity factors, as well as for the development of vaccines based thereon [8–11]. However, according to the literature data, the ASFV population, even within the same geographic region or a large disease outbreak area, may not be homogeneous as regards its biological properties and can simultaneously comprise the strains differing in virulence and hemadsorption activity in cell cultures. This is indirectly evidenced by the data from the studies of the isolates recovered from ticks, as well as by the increasing number of seropositive sample detections in boar in Eastern Europe [12, 13]. Obtaining reliable results requires the analysis of properties of the virus material samples that are homogeneous by composition, rather than of the entire heterogeneous population of the virus. Therefore, researchers are facing the task of

isolating clones or pure viral lineages being homogeneous as regards their biological properties.

All the methods used for the isolation of such clones are based on the separation of a heterogeneous virus mixture into individual samples and the determination of their biological properties for the identification of virus subpopulations followed by their isolation and the propagation of single infected cells or viral particles. However, conventional culture-based methods for viral particle cloning are not without certain disadvantages; in particular, they are rather time- and labour-consuming. Thus, in order to simplify and automatize the selection and cloning of virus infected cells, it is appropriate to apply flow cytometry cell sorting. Cell sorting can also be applied to enrich or purify cell preparations within various research activities, and cytometry can also be utilized for absolute cell count determination in a tested sample using special calibration counting particles or by a volumetric method [14–16].

To achieve all these purposes, different methods based on certain physical principles, which can be classified as active and passive, are applied. Active systems typically use external forces (acoustic, mechanical, electric, magnetic and optical) to displace cells for sorting, whereas passive systems use inertial forces, filters with different pore sizes and adhesion mechanisms [16–18].

Fluorescence-activated cell sorting (FACS) allows for the detection and purification of specific cell populations based on their phenotypic markers, such as relative size, granularity, the presence (on their surface or in the space delimited by a cell membrane) of specific clusters of differentiation identified by flow cytometry. This technique enables researchers to get a better insight into the characteristics of the target population without any impact of other cells.

At present, fluorescence-activated cell sorting methods are automated and robust. Modern flow systems are able to analyze and sort over 50,000 particles per second based on various criteria. In conventional FACS systems, fluorescently labeled cells organized in a laminar flow stream pass through a focused laser beam that scatters into one or more photodetectors. Then the fluorescent signal is analyzed to assign a certain cell type to each registered event of interception with the laser beam. After being typed, each single cell can be deposited into an individual well of the culture plate through discrete sorting. A particle is encapsulated into an aerosol droplet that is charged. When a charged droplet passes through charged plates, it is electrostatically sorted [14, 16].

The sensitivity of FACS is so high that it even allows the sorting of single cells for their subsequent sequencing. This technique is, nevertheless, not without disadvantages. Sorting can be hampered when the cells or particles of interest have a high level of autofluorescence. Besides, the binding of fluorescently conjugated antibodies with their specific ligands located outside the cells can alter the functional activity of sorted cells, thus influencing the results of further experiments. Therefore, the methods based on cell assessment by their size and granularity were selected for the primary sorting of the ASFV infected cell culture. And last but not least, the appropriate operation of a flow sorting system requires, in most cases, the involvement of a highly qualified personnel [17].

It should be noted that fluorescence-activated cell sorting can be applied not only for cells, but also for other discrete particles, including intracellular vesicles, and even for individual virions. However, the translation of this idea into practice raises certain difficulties related to the specific features of virion morphology and the configuration of the optical system of the instrument used [18–22].

First of all, the virus should be propagated to sufficient titres in the cell culture, then the infected cells should be destroyed to release virions. Different methods, such as a freeze-thaw cycle, ultrasound sonication, cell membrane lysis or osmotic pressure modification, can be employed to destroy cells [23]. As a result, a suspension containing residual live, dead and dying cells, cell debris of variable size and virions is formed. The fraction of virions will also have a heterogeneous composition, since it will contain both mature virions capable of infecting live cells and immature virions lacking infectivity, as well as destroyed fragments of virions, released viral nucleic acid and incompletely assembled empty viral capsids. The studies of ASF virus will add to this list the mature virions that have acquired a supercapsid envelope made of the cell membrane after complete budding from the cell. Thus, the virus-containing suspension resulting from cell destruction requires further purification from residual cell debris and nonfunctional virions.

In addition to the above, the sorting of individual virions is limited by the specific characteristics of the optical system configuration of the sorter used. In view of the fact that the virion is as small as a few hundreds of nanometres, whereas average eukaryotic cell sizes are from 10 to 50 μm, the detection of an individual virion requires the use of a highly sensitive detection system that is not only able to register even the smallest changes in laser beam brightness caused by a viral particle passage through it, but also has a high signal-to-noise ratio necessary for effective identification of the signals of interest from the background noise, the presence of which is unavoidable during the analysis due to a number of physical factors, in particular the mutual overlapping of fluorescence emission frequencies of the dyes applied, the electronic noise of sensors and the scattering of light encountering water molecules. The use of fluorochrome labelled antibodies to the virus cannot fully solve the problem of individual virion detection, since, due to the small area of the virion surface, only a limited number of dye-conjugated antibodies can physically fit onto it. The size of a mature ASFV virion does not exceed 200 nm. Besides, the said antibodies will bind with their specific ligands located not only on the mature and infectious virions, but also on the surface of empty virions and especially of fragments thereof, which could result in an increased background fluorescence level and hindered detection [22, 24].

In the light of the above, this study was aimed to perfect the procedure for the sorting of single ASFV infected cells from porcine bone marrow (PBM) and continuous African green monkey kidney (CV-1) cell cultures being heterogeneous by composition into the 96-well plates to select the most promising clones of the virus.

MATERIALS AND METHODS

The infection of susceptible cell cultures involved the use of African swine fever virus "ASF/ARRIAH/CV-1" strain prepared by the adaptation of ASF virus "8 No. 2/Odintsovo-02/14" strain subjected to serial

passages in the continuous CV-1 cell culture. This strain shows a moderate infectivity and can accumulate in PBM and CV-1 cell cultures at titres of 6.0 to 7.0 lg $HAdU_{50}/cm^3$; however, its lethality for pigs of any age group does not exceed 37.5%. It is also important to note that this variant of the virus has retained the ability to infect primary porcine cell cultures [25].

The cultivation of "ASF/ARRIAH/CV-1" strain in the primary and continuous cell cultures was performed at the FGBI "ARRIAH" under the laboratory conditions appropriate for the handling of pathogenicity group II–IV agents. Freeze-dried ASF virus ASF/ARRIAH/CV-1 strain was obtained from the State Collection of Microorganism Strains of the FGBI "ARRIAH". The following two cell cultures were used for the works performed: a primary PBM cell culture grown in Eagle's nutrient medium supplemented with 20% (v/v) fetal bovine serum and a continuous CV-1 cell culture grown in Eagle's nutrient medium supplemented with 10% fetal bovine serum.

To prepare an infectious virus-containing fluid, ASF virus "ASF/ARRIAH/CV-1" strain was cultivated in the 25 cm^3 plastic culture flasks at +37 °C. After a 72-hour incubation of the ASFV infected cell culture, the monolayer was harvested using trypsin solution, transferred to centrifuge tubes and centrifuged to pellet the cell debris with subsequent removal of the supernatant and resuspension in the normal saline solution. The prepared samples were used for sorting.

The virus titre in the tested sample was determined with hemadsorption (HAD) test according to the standard procedure. The infectious titre of the virus was calculated according to the Karber or Reed and Muench method and expressed as lg $HAdU_{50}/cm^3$ [25].

To perform polymerase chain reaction (PCR) testing, the "Test System for African Swine Fever Diagnosis with Real-Time PCR Coupled with Fluorescence Detection" (FGBI "ARRIAH") was applied according to the manufacturer's instruction.

The cells were sorted using a properly calibrated MoFlo Astrios EQ sorter (Beckman Coulter, USA).

The discrimination of the so-called cell doublets being the aggregation of two or more cells, especially when testing poorly disaggregated samples, as well as when using higher liquid flow rates for sorting, was performed through the analysis of pulse height, area and width with the appropriate software.

RESULTS AND DISCUSSION

Primary porcine cell cultures are commonly used for ASFV-related studies, since the ASF agent can be reproduced in them without any preliminary adaptation. For this study, a primary PBM cell culture was chosen; it has a high susceptibility to ASF virus, and the virus replication in this cell culture is accompanied by the occurrence of hemadsorption and the destruction of infected cells.

At first, at least 50 thousand events (photodetector responses to changes in laser beam intensity as a result of particle passage) were collected, then cell doublet discrimination was performed, the cell subpopulation was gated (R1) based on forward versus side scatter parameters, and the cells were sorted onto a slide for microscopic examination at 460× magnification. At this stage,

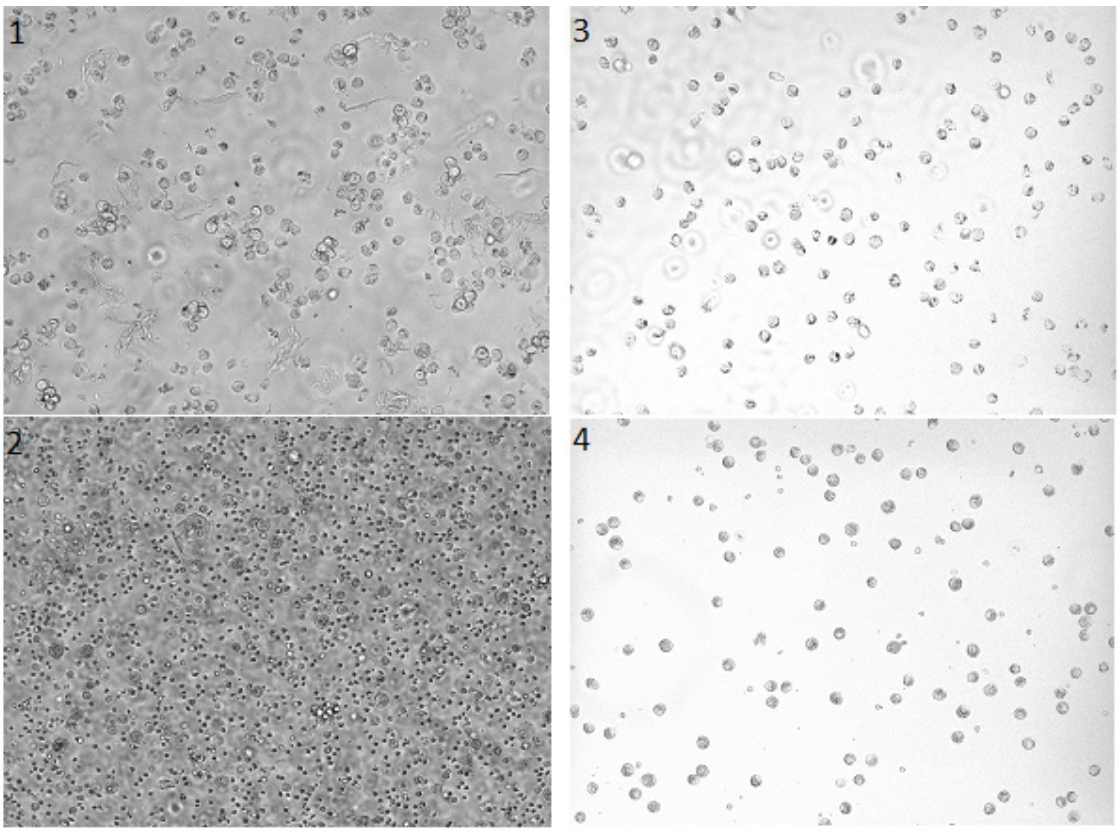

Fig. 1. Cell suspension micrographs taken through an optical microscope (460× magnification) before (1; 2) and after (3; 4) its sorting: upper images – PBM cell culture; lower images – CV-1 cell culture

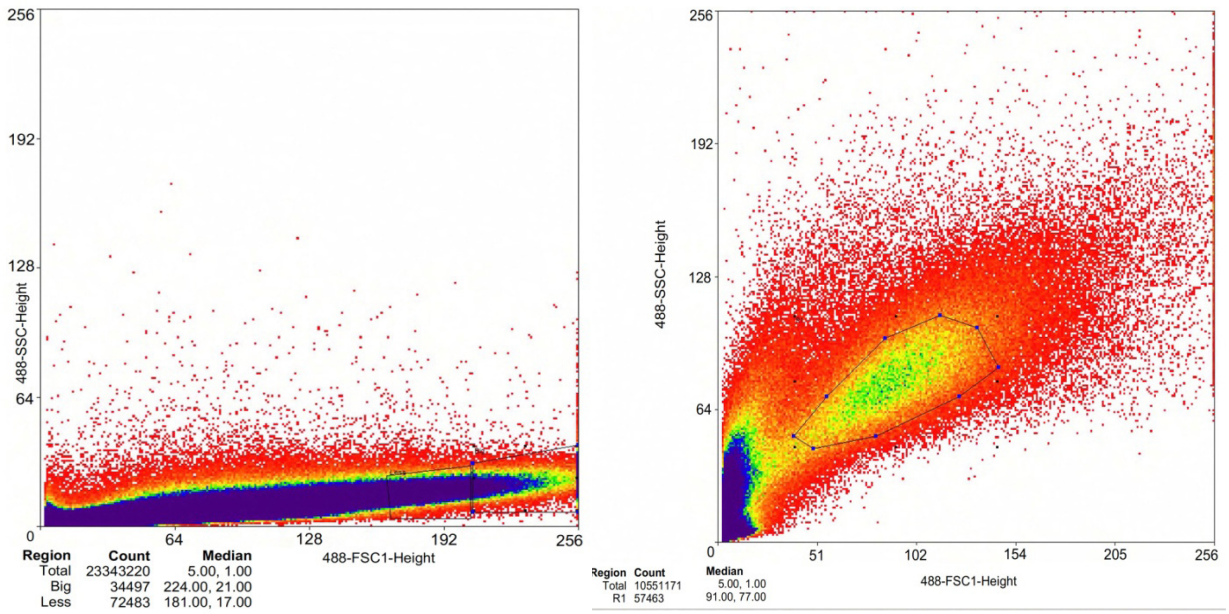

Fig. 2. Side (ordinate) versus forward scatter (abscissa) plots for PBM cells (left image) and CV-1 cells (right image). Gated regions are shown

to accelerate sorting, the sorter was set for lower purity that enables the sorting of droplets containing double or misplaced particles. As the findings presented in Figure 1 show, the resulting suspension has a practically homogeneous cell composition as regards morphological characteristics.

This was followed by single cell sorting into a 96-well culture plate containing Eagle's medium supplemented with 20% bovine fetal serum (0.01 cm^3 per well), 16 wells served as controls. The sorter was set for higher purity so that it could abort droplets containing several particles or an off-centre particle, which corresponds to Single 0.5 sorting mode. The gated region is shown in Figure 2.

In order to isolate and clone new variants of the attenuated ASF virus with stable cultural and biological properties for the further studies of specific features of ASF-associated immunogenesis and the implementation of genetic modifications, sorting of the virus adapted to the CV-1 cell culture was performed using the same sorting parameters.

After PBM cell culture sorting, the HAD test detection rate of ASF positive wells was 30%. Since hemadsorption only occurs in primary cell cultures, CV-1 cells were tested for virus genome fragments with PCR. Based on the PCR test results for the samples prepared using CV-1 cell culture, positive wells were detected in 20% of cases. The detection of negative wells can be attributed to possible sorting of uninfected cells or cells with the virus that has lost its infectivity into these wells. The fact that cell sorting involves the application of mechanical forces to the cells, which increases a cell damage risk, should also be taken into consideration [16].

Besides, aerosol generation at the time of sorting can potentially lead to ASF virus introduction into inappropriate wells. However, the virus was not found in the intact controls of the plate (16 wells) during our experiment.

The virus clones obtained as a result of sorting were propagated in the continuous and primary cell cultures, and this allowed to confirm the presence of the inactivated ASF virus in the selected cells. The virus propagated from single sorted cells to the sufficient amounts was used for a bioassay in naturally susceptible animals.

CONCLUSION

Thus, flow cytometry sorting of cells based on their physical parameters (size and granularity) allows for the preparation of homogeneous enriched cell suspensions. The method makes it possible to clone the virus rapidly and effectively through the sorting of infected cells into a 96-well plate. However, maximum effectiveness would be achieved through using fluorochrome labelled ASFV antibodies to directly sort its extracellular virions, and this is the aim of further work of the specialists of the FGBI "ARRIAH" Reference Laboratory for African Swine Fever.

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