PROTOCOL

Protocol ID:	**NCT05908162**
Brief Title:	**Algorithm for Predicting the Unfavorable Course of Sepsis in Children**
Acronym:	**RRPCEM_SEPSIS**
Official Title:	**To Develop an Algorithm for Predicting the Unfavorable Course of Sepsis in Children Based on a Comprehensive Assessment of Immunological, Biochemical and Molecular Genetic Markers**
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Sponsor:	**The Republican Research and Practical Center for Epidemiology and Microbiology**
Document Date:	**June 06, 2024**

Collection of peripheral blood for the study

We collected 5-10 ml of venous blood under aseptic conditions into two tubes. One tube with anticoagulant (heparin solution or ethylenediaminetetetraacetate (EDTA)), the second tube with blood coagulation activator. The closed vacutainer with blood is inverted several times to mix the blood with the anticoagulant. The patient's blood was delivered to the Immunology and Cell Biotechnology Laboratory within no more than 4 hours of collection.

Investigation of cellular immunity indicators

Sample preparation includes the following steps

- Spreading of antibodies according to the developed panel in test tubes;

- Adding 100 µl of blood to the antibody mixture and mixing thoroughly;

- Incubation for 15 min at +4 - +12°C in the dark;

- Add 2 ml of lysis solution to lyse the erythrocytes, mix the tube contents;

- Incubate for 10 minutes at +16 to +30°C in the dark;

- Centrifuge the tubes (200-300 g - 5 minutes) to precipitate the leucocytes;

- Add 150 µl DPBS and mix.

- Count on a flow cytometer.

Counting of cell samples on a FACSCalibur cytofluorimeter included the following mandatory steps

- Performance control/calibration of the instrument;

- Adjustment of light scattering parameters on the cytogram plotted in FSC\\SSC coordinates;

- adjustment of the 'threshold' parameters on the FSC channel to exclude cell debris from the analysis;

- Adjusting the voltage on the fluorescence channels using an unstained control sample;

- Adjusting the spectral compensation using single stain controls;

- Counting of the test sample at 50,000 leukocytes.

Data were analysed using Flowing Software version 2.5.1 or BD FACSDiva 7.0.

Sample Preparation for the Study of Spontaneous and Stimulated Production of Oxygen Radicals (ROS) by Blood Neutrophils

Sample preparation involves the following steps:

- Labelling (positive '+' - stimulated sample and negative '-' - unstimulated sample) and serial numbering of sample tubes;

- Place the cytometer tubes in a rack and number them;

- Add 1 µl of DHR to the tubes;

- Add 50 µl of RPMI medium;

- Add 50 µl of blood and mix thoroughly;

- Incubate for 10 minutes at +37°C;

- Add 20 µl RPMI to '+' samples only and mix the contents of the tube;

- Incubate for 30 min at +37°C in the thermostat;

- Add 2 ml lysis solution, mix tube contents;

- Incubate for 10 min at +16 - +30°C in the dark;

- Centrifuge the tubes (200-300 g - 5 minutes) to precipitate the leucocytes;

- Add 100 µl DPBS and mix.

Sample preparation for the spontaneous necrosis and apoptosis of peripheral blood cells (PI, Annexin V FITC) assay.

Sample preparation involves the following steps:

- Appropriately label two tubes (stained and unstained sample) for the cytometer;

- Prepare a 1X lysis solution by diluting a 10X stock solution;

- Prepare a single annexin binding buffer by diluting the 10x stock solution;

- Add 1 ml of blood to a 15 ml tube;

- adding 14 ml of lysing solution to the tube containing blood, mixing the contents of the tube;

- Incubate for 10 minutes at room temperature in the dark;

- Centrifuge the tubes (200-300g - 5 minutes) to precipitate the leucocytes;

- Wash the precipitate with 2.5 ml DPBS: add 2.5 ml DPBS, tip to resuspend the cells;

- Centrifuge the tubes (200-300g - 5 minutes) to precipitate the cells;

- Collect the supernatant;

- Dissolve the precipitate in 200 µl of cold single annexin binding buffer;

- Transfer 100 µl of cell suspension to two cytometer tubes;

- diluting the volume of the cell suspension to 500 µl with cwith cold single annexin-binding buffer;

- adding 2.5 µl Annexin V FITC and 2.5 µl PI only to the stained sample tube;

- gentle stirring on a vortex;

- incubation at room temperature for 20 min in a dark place.

- counting on a flow cytometer.

Statistical processing of results

Statistical processing of the data obtained was performed using Statistica version 12 (StatSoft, USA). Values are presented as Me (25 - 75), where Me is the median and 25 and 75 are the interquartile ranges as 25th and 75th percentiles. The normality of the distribution of values was assessed using the Shapiro-Wilk W criterion. Given the lack of normal distribution in the samples studied, non-parametric methods were used to compare groups of data and study correlations. The Mann-Whitney U-criterion was used to compare two independent samples. A significance level of $p < 0.05$ was used as the criterion for reliability of differences between indicators.