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Optimisation of phagocytosis assay in rainbow trout (*Oncorhynchus mykiss*)

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Abstract: A phagocytosis assay is one of the most commonly used functional immunological methods. There are many possible ways of assessing leucocytes and their ability to ingest different particles. The aim of this study was to optimise the phagocytosis assay in rainbow trout (*Oncorhynchus mykiss*) using labelled zymosan particles (Alexa Fluor 488 and Texas Red conjugate). Whole blood was incubated with the particles under different conditions and leucocytes were subsequently isolated by haemolysis in a hypotonic environment. The effect of the different incubation time, temperature, blood volume and dilution on the phagocytic activity was evaluated by flow cytometry. Our experiments showed that the incubation for at least 2 h at 15 °C provided optimal results, while the blood volume and dilution had no significant effect. The optimised assay will be used for the examination of fish health and in further experimental studies.

Keywords: flow cytometry; haemolysis; ingestion; particle; zymosan

Aquaculture, as the fastest-growing food production sector in the world, has great requirements for research in this area, especially in disease diagnostics. An effective way of examining the immune system and its ability to react to stimulants is the application of functional immunological assays. In fish, phagocytosis represents one of the most important defence mechanisms (Uribe et al. 2011), as the non-specific immune response is predominant at low temperatures (Le Morvan et al. 1996). Diseases, pollutants and environmental factors can cause a reduction or elevation in the phagocytic

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activity (Magnadottir 2006; Pikula et al. 2020). Therefore, phagocytosis assays stand amongst the most applied immunological methods used to evaluate the fish health status (Lulijwa et al. 2019).

During phagocytosis assays, an engulfment of particles by phagocytic cells is observed to evaluate their activity. The most commonly used particles include zymosan (prepared from yeast cell walls), bacteria and latex beads (Lulijwa et al. 2019). Phagocytosis of fluorescently labelled particles can be effectively evaluated by flow cytometry. This method offers many advantages, in particular, a rapid examination of a large number of cells and easy differentiation of individual cell populations (Chilmonczyk and Monge 1999). In fish, phagocytic cells mainly comprise neutrophils and monocytes/macrophages (Secombes and Fletcher 1992). However, some phagocytic activity has been recorded in B lymphocytes and thrombocytes as well (Overland et al. 2010; Nagasawa et al. 2014).

There are many different approaches to phagocytosis assays. The head kidney and peripheral blood represent the most common samples (Lulijwa et al. 2019). However, the peripheral blood allows a non-lethal collection (Lawrence et al. 2020). Although leucocytes are usually isolated by density gradient centrifugation, haemolysis in a hypotonic environment, originally published by Crippen et al. (2001), was proven to be a quick, cheap and dependable way of leucocyte separation (Hu et al. 2018). Phagocytosis assays are often used, but incubation conditions vary greatly between different studies. Therefore, the aim of our work was to determine the optimal conditions for this method. The effects of the different incubation time, temperature, blood volume and dilution were evaluated. The differences in the immune response under various incubation conditions were described in previous studies (Minarova et al. 2019; Heger et al. 2020). We hypothesise that this assay is also significantly affected by these factors and its optimisation will improve the examination of the fish immune system by providing more reliable results.

MATERIAL AND METHODS

Fish rearing conditions, sample collection

Rainbow trout (*Oncorhynchus mykiss*; all-female population; total length of 31.28 ± 2.23 cm, average weight of 501.16 ± 134.50 g, 32 fish in total) were kept in a 1 000 l tank of a recirculating system at Mendel University in Brno (Czech Republic).

The average water temperature was 17.93 °C, O_2 8.48 mg/l (i.e., 91.61%), pH 7.11, N-NH_4^+ 0.55 mg/l, N-NO_2^- 0.10 mg/l and Cl^- 136.99 mg/l. The water was filtered by a Nexus 310 biofilter and disinfected by a UV-C lamp. The fish were fed twice a day (BioMar EFICO Enviro 920 Advance) following the supplier's recommendations (BioMar, Brande, Denmark).

Blood samples (1 ml) were collected from the caudal vein into a heparinised (Zentiva, Prague, Czech Republic) syringe and the fish were humanely euthanised by a blow to the head. Samples from eight fish were taken for each of the four individual experiments (defined by the particular incubation time, temperature, blood volume and dilution).

All the experiments were performed in accordance with the EU Directive 2010/63/EU for animal experiments. The national regulations of the Czech Republic were followed (Act No. 246/1992 Coll.) and the experiments were approved by the Branch Commission for Animal Welfare of the Ministry of Agriculture of the Czech Republic (Permission No. MŠMT-19896/2015).

Phagocytosis assay

Whole blood (50 µl) was separately incubated with two types of labelled zymosan particles (5 µl) – Zymosan A (*S. cerevisiae*) BioParticles, Alexa Fluor 488 conjugate (1 mg/ml; Thermo Fisher Scientific, Waltham, USA) or Texas Red conjugate (2 mg/ml). In advance of the assay, the particles were diluted to the required concentration with Hank's balanced salt solution (HBSS; Lonza, Basel, Switzerland) and sonication (3 × 5 s, 20 kHz; Sonopuls GM 3100; Bandelin Electronic GmbH & Co, Berlin, Germany) was performed to homogenise the zymosan suspension and break up aggregates.

The incubation was carried out in a CO_2-free incubator (Memmert, Schwabach, Germany). The control samples were analysed immediately, without any incubation.

During this study, four consecutive experiments were conducted. In the beginning, standard incubation conditions were selected based on pre-experiments: 15 °C and 50 µl of the non-diluted blood

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were considered the default parameters. In the first experiment, three different incubation periods were investigated: 1, 2 and 3 hours. In the second experiment, whole blood was incubated for 3 h and three different incubation temperatures were compared: 4, 15 and 22 °C. The third experiment investigated three different blood volumes (10, 50 and 100 µl), with the zymosan particles added in the same ratio as previously described. In the fourth experiment, the incubation of whole blood was compared with the incubation of the blood diluted with HBSS (2 ×).

After the incubation period, an ethylenediaminetetraacetic acid disodium salt solution 0.5 M (5 µl) (EDTA; Merck, Darmstadt, Germany) was added to inhibit phagocytosis, and leucocytes were isolated by haemolysis in a hypotonic environment (3.6 ml of distilled water for 10 s with the subsequent addition of 0.4 ml of 10X Dulbecco's phosphate buffered saline (10X DPBS; Lonza, Basel, Switzerland).

The isolated cells were washed by centrifugation in DPBS (5 min, 300 *g*, 4 °C; Lonza, Basel, Switzerland), fixed with a cell washing solution [CWS, 100 µl; PBS containing 1.84 g/l EDTA, 1 g/l sodium azide and 4 ml/l gelatine, all from Merck (Darmstadt, Germany)], and forced through a fine nylon mesh to obtain a pure cell suspension. The cell viability was confirmed with propidium iodide (Merck, Darmstadt, Germany), none of the samples showed less than 95% viable leucocytes.

Flow cytometry

A BD LSRFortessa flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was used to detect the phagocytes with ingested particles; at least 70 000 events were recorded for each sample (2×10^6 cells/ml). The data analysis was performed using the BD FACSDiva software, v6.2 (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). After exclusion of the dead (PI negative) cells, the viable granulocytes were gated according to their light scatter properties; another gate was created for lymphocytes (Figure 1). Based on their green (Alexa Fluor 488) or red (Texas Red) fluorescence, the cells with ingested particles were detected. Only 1–2% of the lymphocytes showed positivity and were not included in the results.

Statistical analysis

The normal distribution of the data was determined by the Shapiro-Wilk test. The Mann-Whitney *U* test (for non-parametric data) or Student's *t*-test (for parametric data) was used for the subsequent analyses. To compare more than two groups, the multi-sample median test (for non-parametric data) or Tukey's HSD (honestly significant difference) test (for parametric data) was applied. The means and standard errors of the means (SEM) were calculated; the results were considered significant if $P < 0.05$.

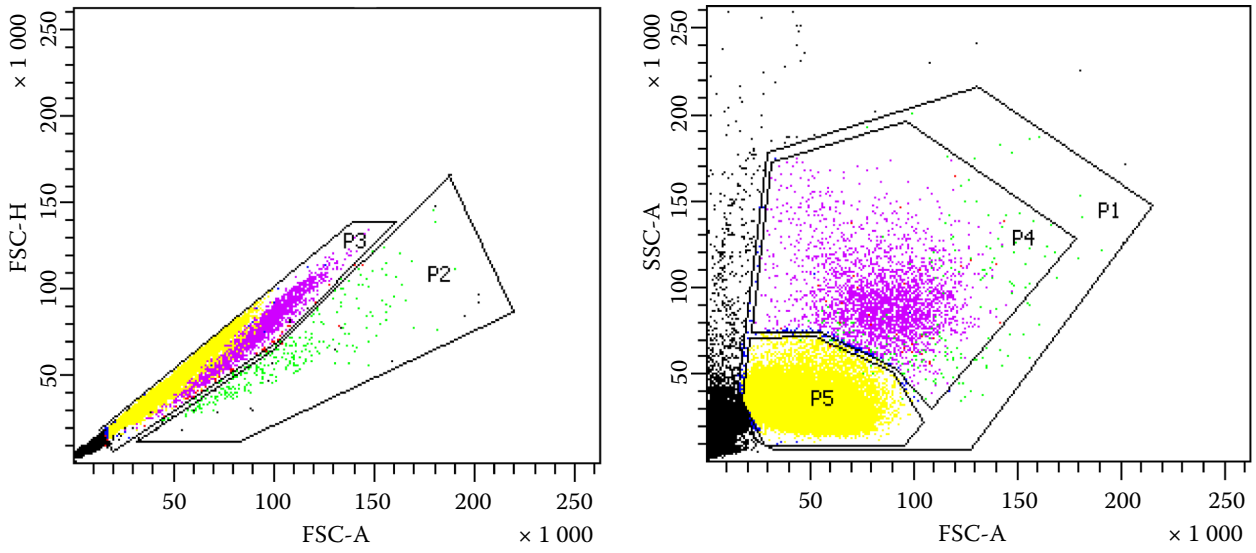

Figure 1. Gating strategy. Leucocytes (P1) were gated according to their forward scatter (FSC) and side scatter (SSC); doublets (P2) were distinguished from the single cells (P3) and neutrophils with monocytes (P4) from lymphocytes (P5)

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RESULTS

Incubation time

The optimal incubation time was investigated. Whole blood (50 µl) was incubated at 15 °C with labelled zymosan particles (Alexa Fluor 488 or Texas Red) for 1, 2 or 3 hours. After the 2- and 3-hour incubation period, a significantly higher percentage (39.1% at 3 h) of phagocytic cells with ingested Texas Red labelled particles was observed compared to the control (Figure 2; $P < 0.01$). Similarly, the highest values (although statistically insignificant and lower than with Texas Red) were detected after the longer incubation period with Alexa Fluor 488 (22.8% at 3 hours). The control values were also lower (6.6%); a relatively high percentage was obtained with the Texas Red control (16.6%).

Incubation temperature

To determine the optimal incubation temperature, whole blood (50 µl) was incubated for 3 h with the zymosan Texas Red labelled particles. The incubation was carried out at 4, 15 or 22 °C. A significantly higher percentage of phagocytes with ingested zymosan particles (30.4%) compared to the control (12.6%) was obtained only with the incubation at 15 °C (Figure 3; $P < 0.05$).

Blood volume

The effect of the blood volume on phagocytosis was investigated. Whole blood (10, 50 or 100 µl) was incubated for 3 h at 15 °C with the labelled zymosan particles (Alexa Fluor 488 or Texas Red; Figure 4). The highest values were obtained with 100 µl of the blood incubated with the Texas Red labelled particles (38.0%) and decreased with the lower blood volumes (30.8% with 10 µl). Alexa Fluor 488 showed the reverse results, the highest percentage was observed with 10 µl (33.5%) and the lowest with 100 µl of blood (24.1%). However, the differences were not statistically significant. The control values with 10 µl were lower (1.3% with Texas Red and 1.1% with Alexa Fluor 488) than the values obtained with the other blood volumes (5.3–6.0%).

Blood dilution

The effect of the blood dilution was determined by the incubation of the diluted (2 ×) and whole blood

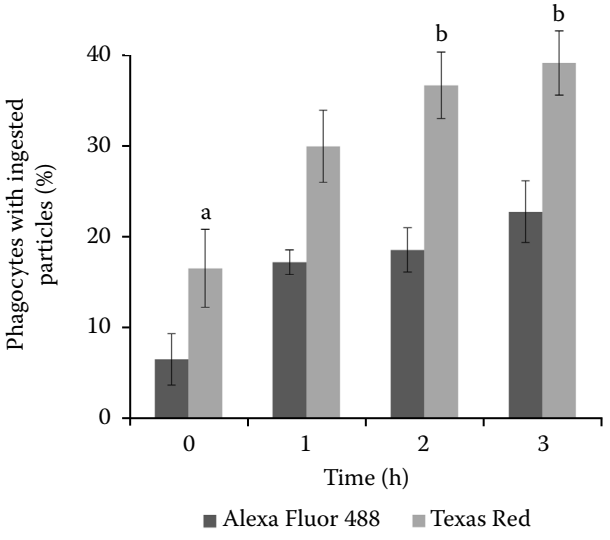

Figure 2. The effect of the incubation time (0 = control; 1, 2 and 3 h) on phagocytosis. Whole blood (50 µl) was incubated at 15 °C with the zymosan Alexa Fluor 488 or Texas Red labelled particles. The values (means ± SEM, $n = 8$) with different letters are significantly different ($P < 0.01$)

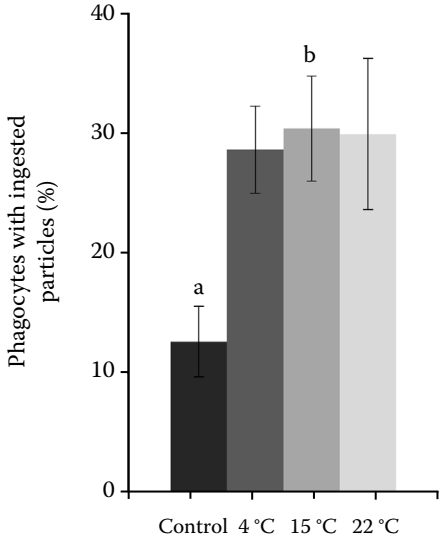

Figure 3. The effect of the incubation temperature (4, 15 and 22 °C) on phagocytosis. Whole blood (50 µl) was incubated for 3 h (control = 0 h) with the zymosan Texas Red labelled particles. The values (means ± SEM, $n = 8$) with different letters are significantly different ($P < 0.05$)

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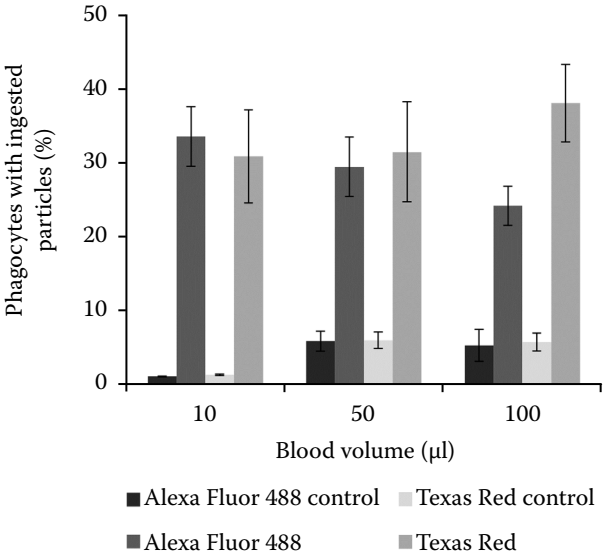

Figure 4. The effect of the blood volume (10, 50 and 100 µl) on phagocytosis. Whole blood was incubated for 3 h (control = 0 h) at 15 °C with the zymosan Alexa Fluor 488 or Texas Red labelled particles. The values represent means ± SEM, n = 8, no significant differences were detected between the blood volumes

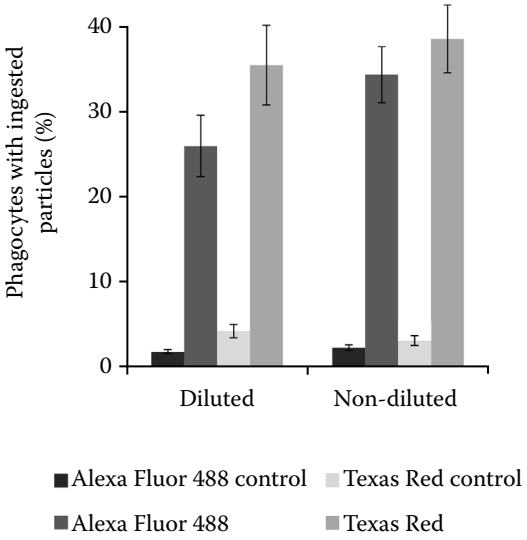

Figure 5. The effect of the blood dilution on phagocytosis. The diluted (2 ×) and whole blood (50 µl) was incubated for 2 h (control = 0 h) at 15 °C with the zymosan Alexa Fluor 488 or Texas Red labelled particles. The values represent means ± SEM, n = 8, no significant differences were detected

(50 µl) for 2 h at 15 °C with the labelled zymosan particles (Alexa Fluor 488 or Texas Red; Figure 5). The non-diluted blood provided better results (34.4% with Alexa Fluor 488 and 38.6% with Texas Red) than the diluted samples (26.0% with Alexa Fluor 488 and 35.5% with Texas Red). However, the differences were not statistically significant.

DISCUSSION

Standardised incubation conditions are required to produce reliable results. The length of the incubation used by different authors in phagocytosis assays varies from 30 min (Sieroslawska et al. 2007) up to 18 hours (Chilmonczyk and Monge 1999). In our study, a significant increase in the phagocytic activity was recorded at 2 and 3 h of incubation. Similarly, Haugland et al. (2012) also observed a higher percentage of lumpsucker (*Cyclopterus lumpus*) leucocytes with ingested beads after longer incubation periods (4 and 8 h) compared to a shorter one (1 hour). Our results indicate that 2 or more hours are optimal for the assay because the increase in the phagocytic activity was significant after this time point. Longer incubation periods provide higher values but may be unnecessary because the following increase is not as significant and the cell viability decreases over time (Crippen et al. 2001). In this part of the experiment, relatively high control values were obtained with the Texas Red labelled particles. This observation can be explained by a higher quantum yield, an attachment of the zymosan particles to the cell membrane (Thiele et al. 2001) or by particle aggregation (Park et al. 2020).

As for the incubation temperature, significantly higher results were detected only at 15 °C compared to the control. Similar (Chilmonczyk and Monge 1999) or higher (Li et al. 2004; Hu et al. 2018) temperatures (20 °C) are commonly used in phagocytosis assays performed in other studies. Corresponding to our observations, Thuvander et al. (1987) obtained better results with the incubation at 19 °C compared to 4 °C. However, higher than optimal temperatures can cause an impairment of the phagocytic activity (Collazos et al. 1995). Our findings suggest that 15 °C, consistent with the rainbow trout (*Oncorhynchus mykiss*) optimum temperature (Bear et al. 2007; Dibble et al. 2018), is the best choice for the assay.

The phagocytic activity was not significantly affected by the volume or dilution of the incubated blood in our experiments. Even though most sal-

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monid studies used the incubation of isolated leucocytes (Lulijwa et al. 2019), our findings showed that comparable results can be obtained with the incubation of whole blood. In addition, whole blood provides more natural conditions (e.g., plasma factors) for the incubated cells, the assay is easier to perform and less time-consuming.

The isolation of leucocytes by haemolysis in a hypotonic environment was proven to be a reliable method in our study. Compared to the isolation by density gradient centrifugation, it is less demanding in terms of time, costs, and laboratory equipment. This technique, in fish originally described by Crippen et al. (2001), offers many advantages and can also be used for a non-lethal examination, and, in this way, contributes to ethically appropriate research (Lulijwa et al. 2019). Hu et al. (2018) reported that peripheral blood leucocytes (PBL) obtained by the hypotonic lysis of erythrocytes had a similar cellular composition to the PBL isolated by the gradient method, with no impairment of their functional capability. Density gradient centrifugation also showed higher erythrocyte contamination and loss of some high-density cells (mostly B lymphocytes and neutrophils), which supports the proposition that the separation of leucocytes by haemolysis in a hypotonic environment is more effective and preferable. Even though gradient centrifugation is still the most frequently used method of leucocyte isolation, new assays are being developed to minimise its negative effects (Korytar et al. 2013).

In our study, a significant increase in the phagocytic activity was detected after the incubation for at least 2 h at 15 °C; no significant effect of the blood volume or dilution was observed. The incubation of whole blood with the subsequent leucocyte isolation by haemolysis in a hypotonic environment was confirmed to be an effective and reliable method. Optimisation of the phagocytosis assay using zymosan particles labelled with Alexa Fluor 488 or Texas Red contributes to the extension of the possibilities for disease diagnostics and improves the assessment of the rainbow trout immune function.

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Conflict of interest

The authors declare no conflict of interest.

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