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Validation and Comparative Analysis of Dihydrorhodamine 123 Oxidative Burst Measurement by Flow Cytometry in Neutrophils: A Study of Two Isolation Techniques and Two Bacterial Strains

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Abstract

Objective: Dihydrorhodamine (DHR) 123 measurement by flow cytometry is widely used to detect neutrophil phagocytosis and oxidative burst activity. Our study aimed to evaluate the performance characteristics of DHR 123 assay results of neutrophils isolated by two different techniques and stimulated with two bacterial strains according to the validation principles.

Material and Methods: The oxidative burst index of neutrophils was measured by flow cytometry using healthy human venous blood samples. Granulocytes were separated by two different density separation methods, Ficoll and dextran sedimentation, and stimulated with two bacterial strains (ATCC 25923 *Staphylococcus aureus* subsp. *aureus* Rosenbach and ATCC 25913 methicillin-resistant *S. aureus*). Flow cytometric measurements were performed at five different time points (0, 10, 20, 30, 60 min). Statistical analysis was performed using GraphPad Prism version 8 software (GraphPad Software, Boston, USA) to assess linearity, precision, and sensitivity and to compare methods, bacterial strains, and incubation times.

Results: Our study showed that isolation by the dextran method was more suitable due to low limits of detection and quantification. Both ATCC strains were suitable for use, but ATCC 25923 may be preferred because the dextran isolation method with strain ATCC 25923 had the lowest limit of detection and quantification. Our data also showed that measurement at 0 and 30 min was appropriate.

Conclusion: Our study contributes to the standardization of functional methods for neutrophil oxidative burst analysis.

Keywords: Flow cytometry, dihydrorhodamine 123, neutrophil, validation study

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Introduction

Neutrophils are the most abundant cells, accounting for 50-70% of all white blood cells. They are critical in the immune response against bacterial and fungal pathogens. They are characterized by their ability to rapidly migrate to sites of infection or injury to eliminate invading pathogens (1). Neutrophils trigger microbicidal mechanisms by engulfing and digesting foreign particles via phagocytosis, secretion of proteolytic enzymes, and antimicrobial peptides (2, 3). Neutrophils also perform a process called respiratory oxidative burst activity, which leads to an increase in reactive oxygen species (ROS) and oxidative stress (3). ROS are composed of superoxide anions (O_{2^-}) and hydrogen peroxide (H_2O_2) and create a highly toxic environment for ingested microorganisms, allowing the bactericidal action of neutrophils (4). Therefore, ROS are ideal targets for investigating neutrophil function.

Defects in neutrophil activity, which can be quantitative or functional, result in weak defense against infection. The functional defect, in which reactive oxidative burst activity fails, is well described in chronic granulomatous disease (CGD) (5). In addition, neutrophil oxidative burst activity may be higher or lower compared to the healthy state during the progression of many diseases, under treatment with certain drugs, or in environmental exposure to certain agents (6-11). The measurement of neutrophil oxidative burst has historically been performed using different techniques to guide clinical practice and research on neutrophil function (12-16). One of these techniques is the widely used flow cytometric dihydrorhodamine (DHR) 123 assay (16). The DHR 123 assay is widely used in clinical immunology to evaluate phagocytic function, particularly for diagnosing CGD and, in some cases, glucose-6-phosphate dehydrogenase (G6PD) deficiency (17, 18). In CGD, the test helps detect nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex dysfunction by assessing neutrophil oxidative burst capacity. DHR 123 test can also distinguish between the genetic forms of CGD (19, 20). Pathogenic variants in the CYBB gene, which follows an X-linked inheritance pattern, are responsible for about 70% of cases, and carriers can be detected with the DHR 123 test (21). The remaining genetic causes follow an autosomal recessive inheritance pattern and involve mutations in NCF1, NCF2, CYBA, NCF4, or CYBC1 (20). While the DHR 123 test is primarily used for clinical diagnosis, the assay can also be adapted for research purposes to study neutrophil responses to various stimuli and clinical conditions.

DHR 123, the reduced form of rhodamine 123, is a commonly utilized fluorescent mitochondrial dye (22). DHR 123 itself is non-fluorescent, but it easily enters most cells. It is oxidized by ROS to the fluorescent rhodamine 123, which accumulates in mitochondrial membranes and exhibits green fluorescence (23). Rhodamine 123 emits light at 488 nm, and a right shift in the histogram is observed in flow cytometric analysis due to strong fluorescence excitation (24). Measuring fluorescence intensity by flow cytometry helps us detect the oxidative burst activity of neutrophils (24). Several factors may influence DHR 123 flow cytometric neutrophil oxidative burst assay results, including sample preparation, neutrophil purification technique, storage time and conditions, DHR 123 concentration, type of stimulant used, pre-measurement incubation time, temperature and pH, laser power and filter settings of the flow cytometry (25-32). The validation and optimization of these factors for the experiment are crucial for obtaining the most accurate results.

Before conducting a DHR 123 neutrophil oxidative burst assay, every step must be considered and carefully planned, as neutrophils are sensitive to physical and chemical interventions in *in vitro* studies and have a short lifespan of 4-6 hours when out of circulation (33). At the beginning of the assay, good selection and application of the neutrophil isolation protocol during the sample preparation phase are essential to obtain a cell population with as high survival and purity without monocyte contamination as possible and to avoid de novo activation and false positive signals (34). For this purpose, “gradient separation”, called the “Ficoll method”, and “dextran sedimentation followed by the density gradient separation”, called the “dextran method”, are commonly used methods for neutrophil isolation. In addition, the chemical or biological agents used in the stimulation and pre-measurement incubation time are also important parameters affecting the test results’ accuracy and sensitivity. Therefore, meticulously identifying and controlling these factors are essential for obtaining accurate and reliable results. Various methods for isolating and stimulating neutrophils have been proposed in the literature; however, there is no consensus on which isolation technique and stimulation type is ideal (27, 35-42). Furthermore, the pre-measurement incubation time, an important part of DHR 123 analysis, may

vary according to different protocols. It is important to optimize and standardize the DHR 123 neutrophil oxidative burst measurement technique between laboratories to achieve accurate results and correctly guide the clinic (43). Validation studies are important stepping stones to this path and help us assess the suitability of different protocols and analysis methods for their intended use (34). To validate an analytical procedure, prior knowledge, data, or experiments are evaluated. According to "ICH Harmonised Guideline: Validation of analytical procedures Q2 (R2)", in the validation process, analytical procedure objectives should be set in the first step, performance characteristics should be clarified based on the objectives in the second step, and validation tests should be performed in the last step (44). Assay validation is a crucial process in which an assay is rigorously tested against specific criteria to confirm its suitability, reliability, and consistency for its intended use. It is essential for accurate research outputs and ensuring the accuracy of measurement results provided to healthcare providers.

In the framework explained above, in our study, we aimed to detect the performance characteristics of the assay results of the DHR 123 test of healthy human neutrophils isolated by two different techniques and stimulated with two different ATCC strains (ATCC 25923 - Staphylococcus aureus subsp. *aureus* Rosenbach and ATCC 25913 - methicillin-resistant *S. aureus* [MRSA]). The results obtained for this purpose were evaluated using validation parameters such as precision (including reproducibility), linearity and analytical sensitivity (limit of detection and limit of quantification). In addition, our research involved comparing two different separation methods, assessing stimulation with two distinct bacterial strains and evaluating incubation and measurement times.

Materials and Methods

Blood Samples

For this study, venous blood samples of five healthy volunteers were drawn into 10 mL syringes pre-filled with sodium heparin (40 IU/mL) and immediately subjected to neutrophil isolation. Voluntary informed consent forms were obtained before the utilization of blood samples from healthy individuals. The Yeditepe University Clinical Research Ethics Committee approved the study on October 18, 2024, with the decision number 2024-KAEK-21/1035.

Bacterial Strains

In this study, two different bacterial strains, *S. aureus* subsp. *aureus* Rosenbach (ATCC® 25923™) and MRSA (ATCC® 25913™), were used to stimulate neutrophils in an oxidative burst assay. Both strains were kindly provided by the Medical Microbiology Department, Faculty of Medicine, Yeditepe University.

Neutrophil Isolation

Two distinct methods were compared within the context of the study. For the "density gradient separation" method, which we called the "Ficoll method" in the context of the study, 3 mL whole blood samples were layered on the lymphocyte separation medium containing Ficoll (v/v: 1/1) (Lymphocyte Separation Medium, Density 1.077 g/mL, Cat-No: LSM-B; Capricorn Scientific, Germany) and incubated for 40 min at room temperature. Supernatants containing the polymorphonuclear cells were transferred to a clean flow cytometry tube for further analysis. For the "dextran sedimentation followed by the density gradient separation" method, which we called the "dextran method" in the context of the study, whole blood samples were mixed by inverting the tubes with dextran solution (3% in 0.9% NaCl, Cat-No: 31392; Sigma Aldrich, USA). After 40 min of incubation, the tubes were centrifuged at 300 g for 5 min, and the collected supernatants were layered on a lymphocyte separation medium containing Ficoll (v/v: 1/1). The tubes were further incubated for 40 min at room temperature, followed by centrifugation at 300 g for 5 min. The supernatants were discarded, and erythrocytes in the erythrocyte-granulocytes pellet were lysed with VersaLyse Lysing Solution (Cat-No: A09777; Beckman Coulter, USA). After 15 min of incubation, the tubes were centrifuged at 300 g for 5 min. The granulocyte pellets were suspended in Dulbecco's phosphate-buffered saline (DPBS) and transferred to a clean flow cytometry tube for further analysis.

Oxidative Burst Measurement in Neutrophils

For stimulating neutrophils, 50 µL of bacterial solution at a concentration of 1 MacFarland was added to 50 µL of neutrophil suspension in each tube. The total volume of the sample was then completed to 500 µL with DPBS, followed by the addition of DHR 123 to tubes (5 µM/test, Cat-No: sc-203027; Santa Cruz Biotechnology, USA). Tubes were incubated at room temperature under dark for 60 min. Mean fluorescence intensity (MFI) values were measured using a DxFLEX flow cytometry system (Beckman Coulter, USA) at 0, 10, 20, 30, and 60 min post-stimulation. The oxidative burst index was cal-

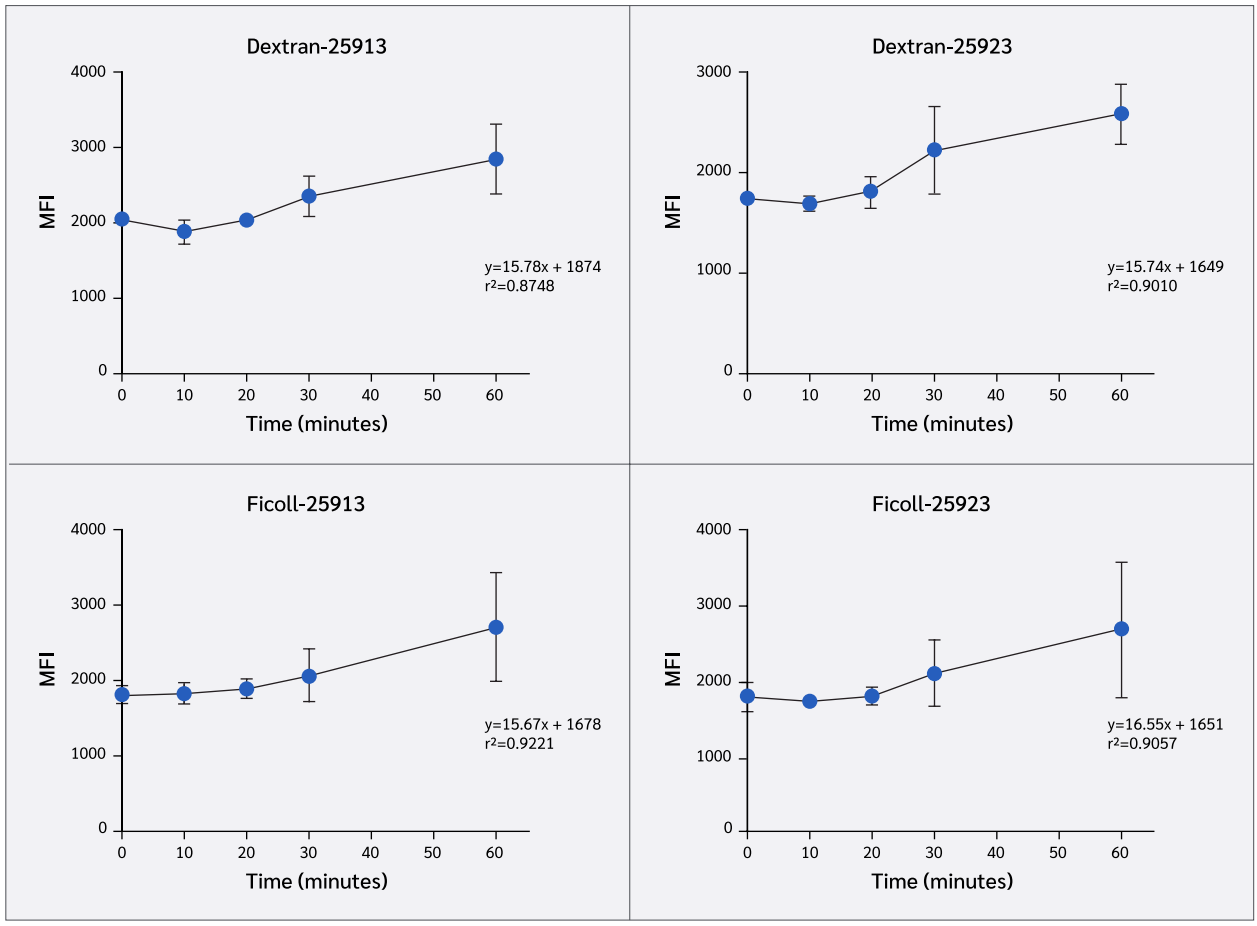

Figure 1. Comparative analysis of protocol performances. The coefficient of determination, denoted as r^2, is computed by squaring the coefficient of correlation (r). This metric represents the percentage of variation in the dependent variable (y) elucidated by the collective influence of all independent variables (x). The higher r^2 value, approaching 1, signifies enhanced linearity in the protocol. The Ficoll isolation method demonstrated the highest level of linearity in the MRSA-25913 protocol.

MFI: Mean Fluorescence Intensity.

culated by dividing the MFI measured at each relevant time point by the MFI at minute 0.

Statistical Analysis and Validation of Analytical Methods

Statistical analyses were conducted using GraphPad Prism version 8 software (GraphPad Software, Boston, USA), incorporating 2-way ANOVA, Pearson correlation, and linear regression methods. The parameters assessed within the framework of this study comprised linearity, analytical sensitivity (including limit of detection [LOD] and limit of quantification [LOQ]), and precision. Two-way ANOVA was performed to compare the efficacy of separation methods and bacterial strains and to determine the optimal incubation duration. Linearity refers to the ability of the method to produce test results that are directly proportional to analyte concentration. When comparing the performance of different protocols, a higher r^2 value approaching 1 indicates greater protocol linearity. The terms "LOD" and "LOQ" mean the lowest concentration at which the analyte can be detected and reliably quantified, respectively. We calculated the detection and quantification limits using the standard deviation of the blank samples (σ) and the slope of the standard curve (S). However, the precision between laboratories could not be analyzed.

Results

Linearity

The Ficoll isolation method used ATCC 25913 MRSA

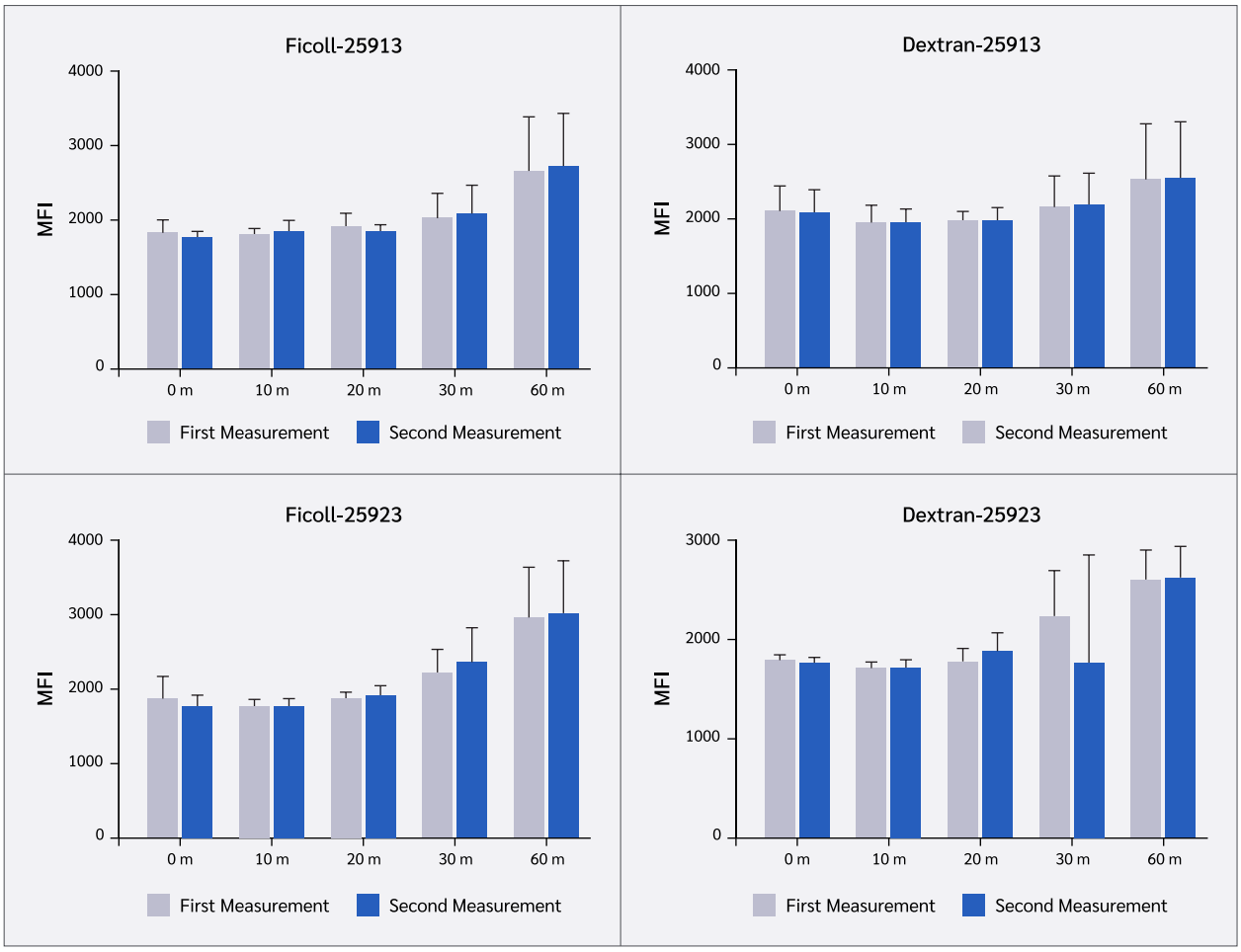

Figure 2. Evaluation of measurement repeatability. Measurement repeatability was assessed by conducting the study in duplicate, revealing no significant differences between the first and second measurements (p>0.05).

MFI: Mean Fluorescence Intensity.

showed the highest linearity.

In this study, the Ficoll isolation method showed the highest linearity with the MRSA-25913 protocol (Figure 1).

Precision

The first and second measurements did not show significant differences.

Precision refers to the degree of agreement between individual test results obtained under identical conditions. The study was conducted in duplicate, and no significant differences (p>0.05) were observed between the first and second measurements (Figure 2, Table S1).

Analytical Sensitivity

The dextran isolation method with strain ATCC 25923 had the lowest LOD and LOQ.

The detection limit was determined using the formula

Table 1. Comparative analysis of limit of detection (LOD) and limit of quantification (LOQ) across protocols.

Protocol	LOD (MFI)	LOQ (MFI)
Ficoll-25923	39.35	119.26
Dextran-25923	5.51	16.68
Ficoll-25913	21.66	65.64
Dextran-25913	60.44	183.14

LOD and LOQ were determined using the standard deviation of blank samples (σ) and slope of the standard curve (S), with the formulas (3.3×σ)/S and (10×σ)/S, respectively. The dextran isolation method using bacteria coded 25923 yielded the lowest limits.

LOD: Limit of detection **LOQ:** Limit of quantification
MFI: Mean fluorescence intensity

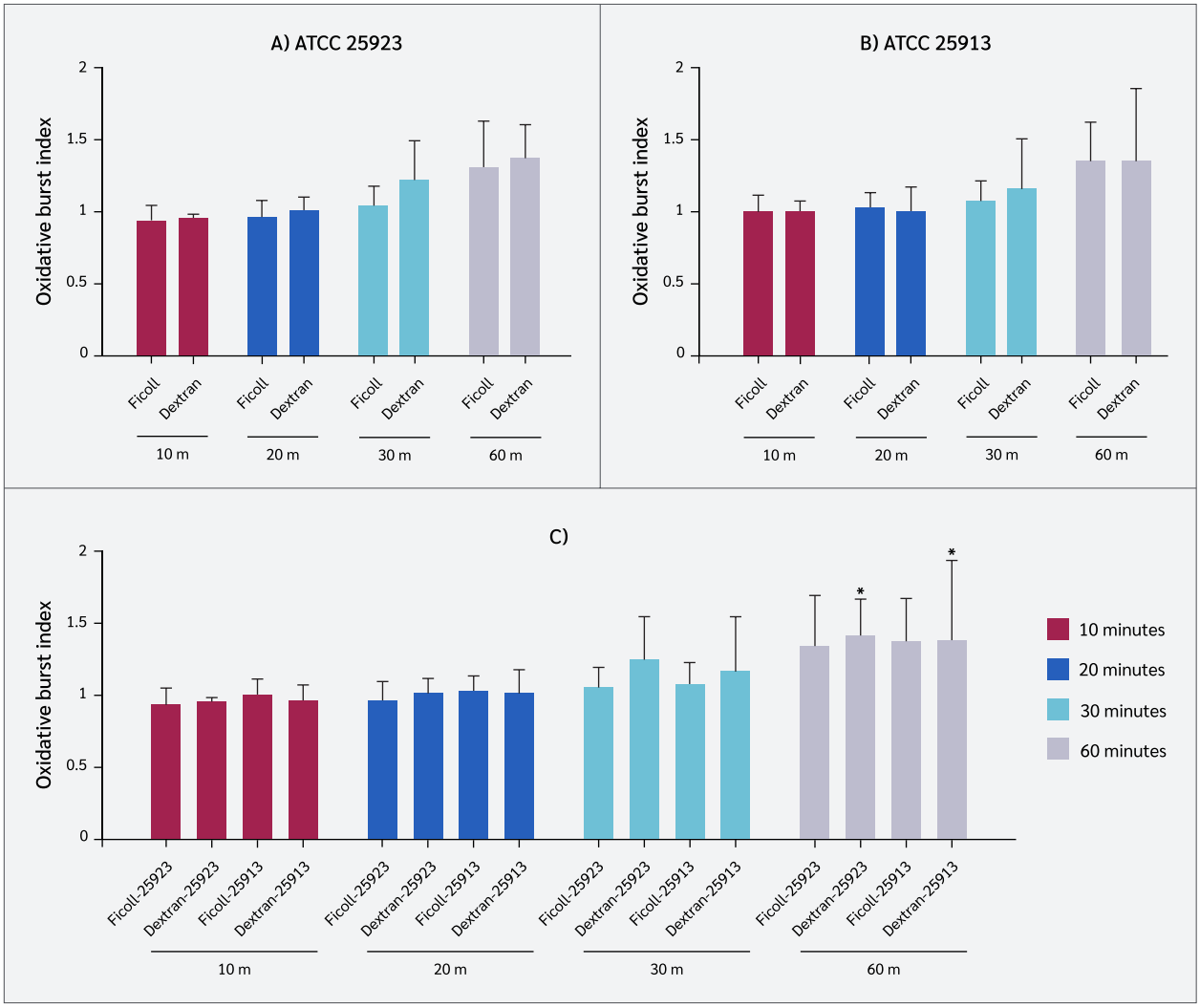

Figure 3. Comparative analysis among protocols and bacterial strains. No significant difference was observed among bacterial strains for stimulation between the periods (p>0.05) **(A, B)**. However, a significant difference was identified between the 10th and 60th minutes for both bacterial strains in neutrophils isolated using the dextran method (Dextran-25913: 10 min. vs. 60 min. *p*=0.04; Dextran-25923 10 min. vs 60 min p= 0.02) **(C)**.

"limit of detection= (3.3×σ)/*S*", and the lowest detection limit was found using the dextran isolation method and bacteria coded 25923 (Table 1). The quantitative detection limit was calculated using the formula "limit of quantification= (10×σ)/*S*", and the lowest limit was obtained using the dextran isolation method with bacteria coded 25923 (Table 1).

Comparison Among Bacteria Between Periods

In the neutrophil oxidative burst test, the index is obtained by dividing the average fluorescence value obtained in the relevant period by the average fluorescence value at minute zero. No significant difference was detected between the bacteria between the periods (*p*>0.05) (Figure 3-A, Figure 3-B, Table S2).

Pre-Measurement Incubation Time

Neutrophils isolated using the dextran method showed a notable change in the oxidative burst index between the 10th and 60th minutes for both bacterial strains. However, measurements at 0 and 30 minutes are appropriate.

A significant difference was found between the 10th and 60th minutes in both bacterial strains in neutrophils isolated by the dextran method (Dextran-25913: 10 min. vs. 60 min. *p*=0.04; Dextran-25923 10 min. vs 60 min *p*=0.02) (Figure 3-C). The literature has reported that

the dextran isolation method activates neutrophils, and the stability of DHR 123 in aqueous solutions needs to be investigated to confirm the specificity of the increase in DHR 123 signal in long-term incubation conditions (30, 45). Thus, the data suggests that measurements at 0 and 30 min are appropriate (Figure 1).

Discussion

The production of ROS is integral to the antimicrobial and physiological functions of phagocytes (1). The precise measurement of this activity is essential, prompting the development of various techniques over the years, each with distinct advantages and limitations (13, 14, 16, 22, 24, 27). Among these, flow cytometric assays have been established to precisely detect oxidative burst activity, traditionally employing nitroblue tetrazolium (NBT) and DHR 123 assays. Emmendörffer et al. (22), in their validation study, identified the DHR 123 method as a highly sensitive alternative to the clinically utilized NBT test for diagnosing chronic granulomatous disease (46). Subsequent studies have established the DHR 123 method as a widely used probe (34, 36, 43). In this study, we used a DHR probe to measure oxidative burst activity.

Purifying human neutrophils for *in vitro* studies is challenging because of their susceptibility to activation during *ex vivo* manipulations (36). Although the DHR 123 test is commonly performed on whole blood in clinical settings, we chose to use isolated neutrophils to ensure a more standardized evaluation of bacterial stimulation effects. This approach minimizes background noise from other blood components, allows more precise gating, and provides better control over the experimental conditions, particularly in assessing bacterial stimulation effects on oxidative burst responses. Quach and Ferrante (45) conducted a comparative analysis of neutrophils purified using the classical 2-step method (dextran sedimentation followed by low-density Ficoll-Hypaque) and the 1-step high-density Ficoll-Hypaque gradient centrifugation. Their findings demonstrated that the 2-step method led to increased CD11b expression, CD62L shedding, adhesion, decreased random migration and chemotaxis, and increased baseline oxidative burst activity. Notably, this effect was not confined to dextran, as Ficoll used for erythrocyte sedimentation also replicated the observed elevation in neutrophil adherence (45). In our study, emphasis was placed on the relative MFI values concerning the zero time point to mitigate the impact of baseline activation on measurement outcomes. DHR 123 measurements were performed at five separate time points following neutrophil stimulation. Additionally, two distinct isolation procedures were employed, and the purified neutrophils were segregated into groups stimulated by two different bacterial strains. This approach allowed us to assess the relative efficacy of the bacterial strains and determine the isolation technique that yielded optimal results under diverse stimulation conditions. Based on the results obtained by validation methods, the superiority of the dextran method over the Ficoll method with low LOD and LOQ was demonstrated.

Smith and Weidemann (47) examined the oxidative burst in human neutrophils stimulated *in vitro* with opsonized zymosan or phorbol myristate acetate (PMA) at the single-cell level using dichlorofluorescein diacetate and DHR 123 as oxidative probes. DHR was the most sensitive probe, with PMA being the stronger stimulus (47). In the diagnosis of CGD, patients with a stimulation index (SI) below 1.5 are considered to have X-linked CYBB deficiency (48). In our study, SI values in healthy controls were lower than expected compared to previously reported values in studies using PMA stimulation (48). This discrepancy is likely due to the choice of stimulation method, as PMA is a potent activator of the NADPH oxidase complex, leading to a strong oxidative burst response. In contrast, bacterial stimulation may elicit a more variable or lower oxidative response, depending on the bacterial component and strain used, opsonization status, and experimental conditions. Smits et al. (49) suggested that a 20-minute incubation period following *Escherichia coli* stimulation is optimal for testing polymorphonuclear neutrophils (PMN) activity in bovine blood (49). In our study, we found that measurements taken at both the 0th and 30th minutes are adequate and optimal for isolated neutrophils stimulated by either of the two bacterial strains: ATCC 25923 (*S. aureus* subsp. *aureus* Rosenbach) and ATCC 25913 (MRSA). Both ATCC strains are suitable for use, but ATCC 25923 may be preferred because the dextran isolation method with strain 25923 has the lowest LOD and LOQ.

Proper sample preparation, neutrophil purification technique, storage time and conditions, DHR 123 concentration, type of stimulant used, pre-measurement incubation time, temperature and pH, laser power and filter settings of the flow cytometry, and optimization of all these factors for the specific experiment are crucial to obtain the most accurate results. Within the existing

literature, investigations pertaining to neutrophil isolation or stimulation methods commonly emphasize the parameters as viability, purity, cellular yield, pre-activation states of cells, and the expression levels of immunological receptors (26, 27, 29, 34, 37, 40, 41, 44-46). As is different from existing literature, our study focused on the validation parameters, including linearity, analytical sensitivity (LOD, LOQ), and precision.

Recently, Krémer et al. (50) compared different neutrophil isolation methods in terms of their efficacy and impact on neutrophil physiology, and the authors revealed that negative immunomagnetic selection yielded neutrophils resembling those in whole blood in terms of their functions. However, this method is more costly than the methods evaluated in our study. Herein, although the dextran method with 30 min stimulation provided similar data compared to the Ficoll method with 60 min stimulation, it is widely accepted that the dextran method yields high-purity neutrophils (40, 45). Additionally, prolonged incubation with DHR 123 may result in non-specific signals (51). Shorter incubation durations with DHR 123 may increase assay sensitivity, but this should be confirmed in future studies.

Study Limitations

The main limitations of this study were the use of only two standard bacterial strains, the limited number of healthy samples, and the use of a single measurement method and device. Evaluating more bacterial strains for clinical trials, increasing the sample size, and comparing the DHR 123 test with other tests based on fluorescence emission detection, such as fluorimetry, would increase the accuracy and reliability of the study. Additionally, analyzing the inter-instrument validity of the test by measuring the samples of the same individuals in another flow cytometry device is recommended. Future studies should address these limitations and compare data from a more comprehensive sample.

Conclusion

Our study revealed that the dextran isolation method was optimal, given its lower LOD and LOQ. ATCC 25923-*S. aureus* subsp. *aureus* Rosenbach was the preferred strain because of its lowest LOD and LOQ using this method, whereas ATCC 25913 MRSA remained suitable. Furthermore, our data suggested that measuring at 0 and 30 min was appropriate.

Our study provided a detailed overview of the DHR 123 flow cytometric assay for measuring neutrophil oxidative burst, the critical role of isolation methodologies, stimulation protocols, and temporal considerations in ensuring the accuracy of the results. By addressing key technical questions, our research significantly contributes to the ongoing efforts to identify ideal methods for studying neutrophil oxidative bursts, benefiting both research and clinical applications. In future studies, exploring alternative methods to isolate neutrophils, examining additional bacterial strains, testing different stimulants, conducting more extensive validation studies, and exploring potential diagnostic applications will further enhance our understanding and application of these assays.

Ethics Committee Approval: The study was approved by the Yeditepe University Clinical Research Ethics Committee on October 18, 2024, with the decision number 2024-KAEK-21/1035.

Informed Consent: Informed consent was obtained from all participants.

Peer-review: : Externally peer-reviewed

Author Contributions: Concept – CP, BA, AOG, GYD; Design – CP, BA, AOG, GYD; Supervision – AOG, GYD; Fundings – GYD; Materials – CP, BA, AOG, GYD; Data Collection and/or Processing – CP, BA, AOG, GYD; Analysis and/or Interpretation – CP, BA, AOG, GYD; Literature Review – CP, BA, AOG, GYD; Writer – CP, BA, AOG, GYD; Critical Reviews –CP, BA, AOG, GYD.

Conflict of Interest: The authors declare no conflict of interest.

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