FLOW CYTOMETRY AS A RAPID TEST FOR DETECTION OF TETRACYCLINE RESISTANCE DIRECTLY IN BACTERIAL CELLS IN MICROCOCCUS LUTEUS

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ABSTRACT
Correct effective doses of antibiotics are important in the treatment of infectious diseases. The most frequently used methods for determination of the antibiotic susceptibility of bacterial pathogens are slow. The detection of multidrug-resistant bacteria currently relies on primary isolation followed by phenotypic detection of antibiotic resistance by measuring bacterial growth in the presence of the antibiotic being tested. The basic requirements for methods of detection of resistance to antibiotics include speed and accuracy. We studied the speed and accuracy of flow cytometry for the detection of tetracycline resistance in the Gram-positive bacteria Micrococcus luteus. Detection of cell viability and reliability of antibiotic resistance was carried out on the Guava EasyCyte flow cytometer (Merck Millipore, Germany) with SYBR Green and PI dyes. M. luteus was exposed to tetracycline (at 30, 90, 180 and 270 µg.mL⁻¹) over 24 hours. Concentrations of live and dead cells were measured after 4 and 24 hours of incubation. The results revealed that the use of mixed dyes PI and SYBR Green allowed the division of cells into large subpopulations of live and dead cells and the DNA of destroyed cells. After 4 h exposure to tetracycline 30 µg.mL⁻¹, the subpopulation of live cells decreased by 47% compared to the positive control. Tetracycline at 90 µg.mL⁻¹ decreased the subpopulation of live cells by 59% compared to the positive control. A continued increase in concentration caused a shift in the population and an increase in dead cells, indicating damage to the cells of the microorganism. Incubation of M. luteus with 180 and 270 µg.mL⁻¹ tetracycline decreased the subpopulation of live cells by 82% and 94%, respectively, in comparison with the positive control. After incubation with 30 µg of tetracycline over 24 h the number of living cells decreased by 70% in comparison with the positive control. Tetracycline treatment (90 µg.mL⁻¹ for 24 h) killed 71% of cells. After exposure to 90 µg.mL⁻¹ tetracycline 29% cells were viable. The viability of living cells was confirmed by a microbiological test.

Keywords: antibiotics; flow cytometry; antibiotic resistance; Micrococcus luteus

INTRODUCTION
Antibiotics are one of the most beneficial discoveries in medicine and public health. However, the use, overuse and misuse of these drugs have led to increases in antibiotic-resistant bacterial infections. Antimicrobial resistance (AMR) poses a serious global threat of growing concern to human, animal and environmental health. This is due to the emergence, spread, and persistence of multidrug-resistant bacteria (Davies and Davies, 2010). The O’Neill Review on Antimicrobial Resistance estimated that 700,000 people die from infections due to resistant organisms every year, and by 2050 AMR will surpass cancer as a cause of death (O’Neill, 2016). Resistance to so-called critically important antibiotics used in medicine is of special concern (Bataeva and Zaiko, 2016).

Fast, accurate antibiotic susceptibility testing is a critical need in addressing escalating antibiotic resistance, since delays in identifying multidrug-resistant organisms increase mortality (Kadri et al., 2018).

Standard methods of detection of antibiotic sensitivity are labor- and time-consuming. Detection of multidrug-resistant bacteria currently relies on primary isolation followed by the phenotypic detection of antibiotic resistance by measuring bacterial growth in the presence of the antibiotic being tested. These conventional methods take a minimum of 24 hours to obtain results after the pure culture is isolated (the analysis typically lasts up to 72 hours) (Akhmaidinova, Lavrinenko and Belyayev, 2017). Working out express diagnostic methods is of importance, and currently, studies are made in various directions (Wang et al., 2010). One of these directions is the use of flow cytometry (FC) for the detection of microorganism viability and resistance. Flow cytometry was adopted for microbiological purposes almost 40 years ago, and the usefulness of this method for...
the identification of microbial pathogens directly in clinical samples or the detection of specific antibodies in serum has been well studied (Álvarez-Barrientos et al., 2000), as has its use in the study of antimicrobial activity of some animal-generated antimicrobial substances (Kotenkova and Polishchuk, 2019).

The quantitative assessment of prokaryotic viability is essential, especially for the confirmation of the activity of novel antimicrobial substances (Kotenkova et al., 2019). Flow cytometry can be used for the analysis of the individual population of cells; therefore, it can provide essential information about bacterial antibiotic resistance. Recently, investigations of bacterial antibiotic resistance seem to be most relevant in the clinical environment because of the problems in finding effective therapies. In comparison with traditional diagnostic methods, FC allows results to be obtained much more quickly (Álvarez-Barrientos et al., 2000). Currently, there are reported experiences of antimicrobial susceptibility tests by FC (Woźniak-Kosek and Kawiak, 2005; Faria-Ramos et al., 2013), but despite the significant progress of clinically significant protocols of FC application, there is insufficient scientific information on its continuous use in microbiology.

This manuscript reports the results of the use of FC with the dyes SYBR Green and PI for rapid assessment of cell viability and antibiotic resistance of the Gram-positive bacteria *Micrococcus luteus*.

**Scientific hypothesis**

There is a considerable need for new techniques that enable quick and specific diagnosis of pathogens resistant to antibiotics to guide correct treatment and to slow the development of resistance. Flow cytometry can provide quick essential information about the resistance to antibiotics of pathogenic microorganisms.

**MATERIAL AND METHODOLOGY**

*Micrococcus luteus* ATCC 4698 from American Type Culture Collection was used.

**Sample preparation**

**Positive, negative and mixed controls of Micrococcus luteus ATCC 4698**

*Micrococcus luteus* ATCC 4698 strain was obtained from the State Research Center for Applied Biotechnology and Microbiology (Obolesn, Moscow region, Russia). Cultures were grown on slanted Trypticase soy agar (TSA, Liofilchem) at 30 °C for 24 h. Cultures from the agar surface were removed with Trypticase soy broth (TSB, Liofilchem) and incubated at 30 °C for 24 h. Cultures were grown to exponential phase (OD_{600} 0.3). The received suspension was used as a positive control.

To obtain negative control, 1 mL suspension was heated at 100 °C for 10 min. To prepare mixed samples, 200 µL of positive control (live cells) and 200 µL of a negative control (dead cells killed by heating) were used.

**Preparation of antibiotics**

Cultures were grown to early exponential phase, at which time tetracycline (Sigma-Aldrich, USA) was added in various concentrations (30 µg.mL^{-1}, 90 µg.mL^{-1}, 180 µg.mL^{-1} and 240 µg.mL^{-1}). Incubation was carried out at 37 °C for 24 h. Concentrations of live and dead cells were measured after 4 and 24 h of incubation with the antibiotic.

**Flow cytometry analysis protocol**

SYBR Green stock solution was prepared by dissolving 5 µL of SYBR™ Green I Nucleic Acid Gel Stain, 10,000 X concentrate in DMSO (Thermo, USA) in 495 µL of deionized water. A PI working solution was prepared by dissolving 1 mg PI (AppliChem, Germany) in 1 mL of deionized water immediately before the study.

A volume of 975 µL of 1X PBS (Santa Cruz, USA) was mixed with 10 µL of SYBR Green (Thermo, USA) and 5 µL PI (AppliChem, Germany), shaken vigorously, then 10 µL of *M. luteus* ATCC 4698 was added and mixed. The samples were incubated in the dark for 15 min and green and red fluorescence signals measured on a Guava EasyCyte flow cytometer (Merck Millipore, Germany) for up to 5000 events.

**Microbiology test**

Verification of cell viability was obtained by microbiological test by transferring 100 µL of each concentration onto a nutrient agar plate (TSA, Liofilchem). All plates were incubated for 3 – 5 days at 30 °C. The growth on nutrient agar was evaluated by the presence of viable cells in the sample.

**Statistical analysis**

Measurements were repeated three times. STATISTICA 10.0 software was used for statistical analyses. The results were calculated as mean ± standard deviation (M ±SD). Significant differences (comparison with positive control) were tested by one-way ANOVA Dunnet test. Differences with *p*-values less than 0.05 were considered as statistically significant.

**RESULTS AND DISCUSSION**

Routine techniques for the detection of resistance to antibiotics are based on a phenotypic study in which microbial growth is observed in the presence of different antibiotics. They yield results in not less than 24 h. In the last two decades, faster AST methods, such as PCR-based tests (Barken et al., 2007) and mass spectrometry-based methods (Opota et al., 2015; Trip et al., 2015) have been developed. However, they do not always provide relevant information on antibiotic susceptibility. For example, PCR-based tests rely on the detection of resistance mutations and genes. However, bacteria lacking resistance mutations and genes may still be able to tolerate and survive antibiotic treatments by utilizing many other mechanisms, some of which are non-genetic (Javid et al., 2014; Sanchez-Romero and Casadesus, 2014). Changes in bacterial physiology caused by antibiotics can be detected using FC and fluorescent viability markers, as has been demonstrated by numerous studies (Gant et al., 1993; Walberg et al., 1997; Gauthier et al., 2002; Ambriz-Avina et al., 2014).
M. luteus was the object of this study. It is a Gram-positive microorganism with cell walls consisting of two polymers, i.e., peptidoglycans (Schleifer and Kandler, 1967) and teichuronic acids (TUAs) (Hase and Matsushima, 1972).

M. luteus can form dormant structures that allow for increased survival under stress. So, cold (4 °C), dryness (2.5% humidity) and starvation increase the survival of M. luteus (Casida, 1980), possibly by creating unfavorable growth conditions, and thereby inducing dormancy (Dib et al., 2013). Thus, cells can survive long periods under adverse environmental conditions (Kaprelyants and Kell, 1993), which can allow the evaluation of the gradual transition of cells from living to die over time. PI and SYBR Green dyes can identify stained cells in different large subpopulations. Resistant organisms are more difficult to kill than susceptible organisms, and the SYBR Green/PI assay can distinguish resistant and susceptible categories based on the number of residual viable cells after drug treatment (Feng et al., 2014).

Figure 1 presents the staining patterns of M. luteus control samples. Positive control M. luteus ATCC 4698 (live cells) displayed green fluorescence, and the corresponding counted events are located in the upper left square of the plot. Negative control (dead cells) displayed red fluorescence, and all counted events are located in the lower right square of the plot.

After 4 h exposure of M. luteus to tetracycline (at 30 µg.mL⁻¹, 90 µg.mL⁻¹, 180 µg.mL⁻¹ and 270 µg.mL⁻¹) the mix of dyes identified the emergence of large subpopulations of live and dead cells and the DNA of destroyed cells (Figure 2).

In Figure 2 the red marker corresponds to live cells, blue to dead cells, and purple to the DNA of destroyed cells. There is an intensification in the displacement of the population of living cells beyond the red marker towards the blue, which indicates dead cells. Since PI and SYBR Green are DNA-binding dyes, it is possible to see a third population on the cytogram – the DNA of destroyed cells. The increase in the number of cells inside the blue marker shows a rapid decline in the numbers of viable bacteria after the addition of antibiotics.

Results of the FC analysis of M. luteus after 24 h incubation with various concentrations of tetracycline are presented in Figure 3. The difference in relative mean fluorescence intensity between live and dead cells increased further following 24 h of treatment. A further decrease in the number of living cells from the initially treated cells was noted, and the presence of a large amount of debris indicated that cell lysis was occurring.

Figure 1 Result of flow cytometry analysis of positive, negative controls and mixed sample of Micrococcus luteus ATCC 4698 without antibiotic influence.

Figure 2 Results of flow cytometry after exposure of M. luteus to tetracycline (at 30 µg.mL⁻¹, 90 µg.mL⁻¹, 180 µg.mL⁻¹ and 270 µg.mL⁻¹) during 4 h incubation.
After 4 h exposure to tetracycline at 30 µg.mL\(^{-1}\), the subpopulation live cells decreased by 47.0% compared to the positive control (Table 1). Tetracycline solution at 90 µg.mL\(^{-1}\) decreased the subpopulation of live cells by 59.0% compared to the positive control.

A continued increase in concentration caused a shift in the population and an increase in dead cells, indicating damage to the cells of the microorganism. So, incubation of \(M. \) luteus with 180 µg.mL\(^{-1}\) and 270 µg.mL\(^{-1}\) tetracycline decreased the subpopulation of live cells by 82.0% and 94%, respectively, in comparison with the positive control. The viability of living cells was confirmed by a microbiological test at all concentrations of the antibiotic.

This completely coincided with the results of the cytometric analysis. In analogical research, FC was used for detecting resistant \(E. \) coli strains. Cytometry showed acceptable results on the model of \(E. \) coli. Relative accuracy was 88.8%, sensitivity – 85.7%, specificity 88.8%, and Cohen’s kappa test showed a value of 0.524 (Akhamaldinova, Lavrinenko and Belyayev, 2017).

Cytometry analysis showed a decline in the numbers of viable bacteria after 24 h incubation of \(M. \) luteus with antibiotics. Incubation with 30 µg of tetracycline decreased the number of stained living cells by 70% in comparison with the positive control. Tetracycline treatment at 90 µg.mL\(^{-1}\) for 24 h killed 71.0% of cells. So, after exposure to 90 µg.mL\(^{-1}\) tetracycline, 29.0% of cells were viable.

The viability of living cells was confirmed by a microbiological test. Incubation on the plate was increased to 5 days. A study by Mukamolova et al. (1998) reported that the viability of \(M. \) luteus cells was restored after some 96 h incubation of starved cells in a resuscitation medium.

After 24 h exposure to tetracycline (180 µg.mL\(^{-1}\) and 270 µg.mL\(^{-1}\)) the majority of the cells in the population had received antibiotic-induced damage. The number of stained living cells decreased by 89% and 98.9%, respectively, in comparison with the positive control (Mukamolova, Kaprelyants and Kell, 1998). The viability of the remaining living cells was confirmed only at a tetracycline concentration of 180 µg.mL\(^{-1}\). Such cell survival can be explained by the ability to transition to cell dormancy in unfavourable growth conditions (Dib et al. 2013). In the study by Nikitushkin et al. (2016), it was reported that in response to unfavourable growth conditions nonsporulating mycobacteria transform into the dormant state with the concomitant formation of specialized dormant forms characterized by low metabolic activity and resistance to antibiotics. This because \(M. \) luteus secretes a small protein called Rpf, which has autocrine and paracrine signaling functions and is required for the resuscitation of dormant cells (Nikitushkin, Demina and Kaprelyants, 2016).

Dormancy is a protective state that enables bacteria to survive antibiotics, starvation and the immune system. Dormancy comprises different stages, including persistent and viable but nonculturable (VBNC) states that contribute to the spread of bacterial infections (Mali et al., 2017). Food products are a source of antibiotic-resistant pathogenic bacteria, a way for the transmission of antibiotic-resistant ‘food’ pathogens through the food chain to humans (Zaiko et al., 2019). There is an ongoing increasing antibiotic resistance crisis and new drugs and antibiotics are urgently needed to combat life-threatening antibiotic-resistant infections (Feng et al., 2018).

**Table 1** The results of measuring the number of cells by flow cytometry.

| Time incubation | K \( \leftrightarrow \) | Tetracycline | 30 µg.mL\(^{-1}\) | 90 µg.mL\(^{-1}\) | 180 µg.mL\(^{-1}\) | 270 µg.mL\(^{-1}\) | \(10^5\) cells.mL\(^{-1}\) |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | total | live | dead | total | live | dead | total | live | dead | total | live | dead | total | live | dead |
| **4 hours**     |       |      |      |       |      |      |       |      |      |       |      |      |       |      |      |
|                 | 5.34a | 5.20 | 0.14 | 3.07bc | 2.80 | 0.27 | 2.84b | 2.10 | 0.74 | 2.40b | 0.90 | 1.5  | 1.50b | 0.30 | 1.20 |
| **24 hours**    | 22.28a | 22.00 | 0.28 | 3.30bc | 1.60 | 1.70 | 3.40b | 1.50 | 1.90 | 2.58b | 0.58 | 2.0  | 2.06b | 0.26 | 1.80 |

Note: * – significant differences of experimental (tetracycline) doses compared with positive control (\(p < 0.05\)).
Fast and accurate antibiotic susceptibility tests can significantly reduce mortality rates and reduce financial costs (Barenfanger et al., 1999). Furthermore, the necessity of rapidly prescribing an initial empirical antimicrobial treatment while waiting for the susceptibility test results from time-consuming standard methods frequently leads to inappropriate treatments (Ibrahim et al., 2000).

As to future work, the same strategy may usefully be applied to other microorganisms (including pathogens in difficult food matrices and other antibiotics. However, the present work shows that one may indeed expect to be able to determine antibiotic susceptibility by FC methods. This could be a very useful tool in the fight against antimicrobial resistance.

CONCLUSIONS

The current conventional methods for the determination of minimal inhibitory concentration (MIC) rely on the growth of the test organism in the presence of the antibiotic, which can be time-consuming depending on the growth speed of the organism, ranging from days for fast-growing bacteria to weeks for slow-growing bacteria. We reported a rapid and novel antibiotic susceptibility testing methodology using FC. This study to demonstrate the feasibility of FC used SYBR Green/PI dyes to assay rapidly the viability of cells and to detect reliably the antibiotic resistance of the Gram-positive bacteria Micrococcus luteus.

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