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Optimization of resazurin-based viability staining for quantification of microbial biofilms

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ABSTRACT

The resazurin-based viability staining is often used to quantify viable biofilm cells grown in microtiter plates (MTP). The non-fluorescent resazurin is reduced by metabolically active cells to resorufin which is fluorescent. The amount of fluorescence generated is related to the number of viable cells present. Unfortunately, the linear range of the method is restricted and the lower limit of quantification is approximately 10^6 colony forming units (CFU) per biofilm. The goal of the present study was to optimize this method to broaden its applicability. We added fresh growth medium and resazurin to mature *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Burkholderia cenocepacia* and *Candida albicans* biofilms formed in MTP. Subsequently, the increase in resazurin-based fluorescence was followed over time and we determined the time needed to reach a specific value of fluorescence as well as the time to reach the maximum fluorescence. These time points correlate with the number of viable cells that were initially present and results were compared to plate counts. Using these alternative readouts, we were able to extend the linear range from 10^6–10^8 to 10^3–10^8 CFU per biofilm, meaning that lower numbers of viable cells can be measured and the effect of anti-biofilm treatments can be quantified more accurately. Moreover, this approach is less expensive and less laborious than conventional plating techniques.

1. Introduction

Conventionally used antibiotics are active against planktonic cells that cause acute infection, but often fail to completely eradicate biofilms leading to persistent infections (Bjarnsholt et al., 2013; Costerton et al., 1999). Biofilms are cell communities embedded in a highly hydrated self-produced extracellular matrix and are attached to biotic or abiotic surfaces. They show inherent tolerance and higher resistance to antibiotic treatment than their planktonic counterparts (Bjarnsholt et al., 2013; Drenkard, 2003; Hoiby et al., 2010). Therefore, there is an urgent need to develop novel anti-biofilm agents and to do so, good model systems and quantification methods are indispensable.

Several direct and indirect methods are used to quantify biofilms in the static microtiter plate (MTP) model system (Coenye and Nelis, 2010), each with its own advantages and disadvantages. Plating is a common quantification method that allows the determination of the number of culturable cells. Selective conditions can be used and isolates are available for further research. Furthermore, absolute cell numbers are obtained, and both very low and very high numbers of microorganisms can be accurately quantified (Hazan et al., 2012). However, plating requires detachment and dispersal of the biofilm cells to obtain a homogenous cell suspension and scraping, vortexing and/or sonication is used for this purpose (Pettit et al., 2005). The number of cells will be underestimated if the dispersing is not done accurately. Moreover, cells will not grow and form visible colonies if they are in a state of starvation or under stress (Freitas et al., 2013; Shen et al., 2010). In addition, since plating is time-consuming and labor-intensive, it is not suitable for high-throughput screening. The number of microorganisms can also be estimated indirectly using several staining methods, for example with fluorescein diacetate, tetrazolium salts or resazurin (Peeters et al., 2007). Resazurin (7-hydroxy-3H-phenoxazin-3 one 10-oxide), also referred to as CellTiter-Blue (CTB) or AlamarBlue, is often preferred over other stains as it is easy to use, stable and non-toxic. Metabolically active cells reduce the blue and non-fluorescent resazurin to the pink fluorescent reaction product resorufin. This reduction is proportional to the number of metabolically active cells present. The fluorescence generated can be measured using plate readers (Hamalainen-Laanaya and Orloff, 2012; O'Brien et al., 2000; Pettit et al., 2005). Quantification with resazurin is fast, inexpensive and suitable for high-throughput estimation of the number of metabolically active cells. However, the results have to be interpreted carefully since only relative values are obtained and the resazurin method as conventionally used has a lower limit of quantification of approximately 10^6 colony forming units (CFU) per biofilm (Peeters et al., 2007). The present study evaluates an alternative approach of using resazurin to quantify the number of viable cells in bacterial and fungal biofilms.

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2. Materials and methods

2.1. Strains and culture conditions

Staphylococcus aureus Mu50, *Pseudomonas aeruginosa* PAO1 and *Burkholderia cenocepacia* LMG 16,656 were cultured on Mueller Hinton agar plates (MH, Lab M, Lancashire, UK) and *Candida albicans* SC5314 on yeast-peptone-dextrose agar plates (YPD, BD, Franklin Lakes, NJ). From these pure cultures, overnight suspensions were made by inoculating 40 ml MH broth for the bacteria and 40 ml YPD broth for *C. albicans* with a loopful of microorganisms. All strains were grown aerobically at 37 °C.

2.2. Antibiotics and antiseptics

The following antibiotics and antiseptics were used: tobramycin (TOB, TCI, Tokyo, Japan), ciprofloxacin (CIP, Sigma, Bornem, Belgium), vancomycin hydrochloride hydrate (VAN, Sigma), amphotericin B (AMF, Sigma), miconazol (MICO, Certa, Waregem, Belgium), chlorhexidin digluconate (CHX, Fagron, Waregem, Belgium), cedium (CED, Qualiphar, Bornem, Belgium), clindamycin (CLIN, Certa) and ozonated olive oil (O_3, Labo Althea, Kapelle Op Den Bos, Belgium). All compounds were dissolved in physiological saline (PS, 0.9% (w/v) NaCl), except for CHX which was dissolved in water of standard hardness (WSH) and MICO which was dissolved in dimethylsulfoxide (DMSO, Sigma); and subsequently diluted in PS. Where necessary, solutions were filter sterilized (0.22 μm, Whatman, Dassel, Germany) prior to use. Treatment with ozonated olive oil was performed as described in Brackman et al. (2013).

2.3. Biofilm formation in 96-well microtiter plates

Overnight suspensions were adjusted with MH broth for the bacteria and YPD broth for *C. albicans* to an optical density (595 nm) of 0.05 for *B. cenocepacia*, 0.2 for *P. aeruginosa* and *S. aureus* and 0.5 for *C. albicans*. These optical densities correspond to approximately 5×10^7 cells/ml. A hundred microliters of the diluted cell suspensions were transferred to the wells of a polystyrene round-bottomed 96-well microtiter plate (MTP, SPL Lifescience, Korea) and then incubated at 37 °C. Ten wells filled with sterile medium served as blank control. Medium was removed after 4 h and biofilms were rinsed with PS to remove non-adhered cells. Fresh medium was subsequently added to the wells and plates were further incubated for 20 h.

2.4. Set-up of calibration curves

To investigate the relation between the number of metabolically active cells in the biofilm and the resazurin-based fluorescence signal obtained, biofilms were formed as described above. After 24 h of biofilm formation, medium was removed, biofilms were rinsed and 100 μl fresh PS was added to the wells. The plates were vortexed (5 min) and sonicated (5 min) (Branson 3510; Branson Ultrasonics Corp., Danbury, CT) and the entire content of the wells was collected. This process was repeated twice in order to harvest all biofilm cells. Serial 10-fold dilutions of this suspension were made in PS and 50 μl of each dilution was added to the wells of a round-bottomed sterile 96-well MTP together with 50 μl double concentrated MH medium (5 wells per dilution). Subsequently, 20 μl CellTiter-Blue was added to the wells and the fluorescence (λ_{ex} 535 nm/λ_{em} 590 nm) was measured every 5 min for 20 h using a multilabel microtiter plate reader (Envision; Perkin–Elmer LAS, Waltham, MA). Plates were incubated in the dark at 37 °C between the measurements and fluorescence measured in the blank control was used to correct for background signal. Simultaneously, the number of CFU per biofilm in each dilution was quantified using conventional plating techniques. The time to reach an absolute fluorescence value of 100,000 and the time to reach the maximum fluorescence was determined for each dilution. These time points and the number of CFU present in every dilution were then used to set up the calibration curves. The experiments were performed in triplicate for each strain.

C. albicans is usually grown in media containing relatively high concentrations of glucose because this fermentable carbohydrate enhances fungal growth. However, our preliminary results showed that the use of this medium leads to high background levels of fluorescence, indicating that the medium influences the measurement (data not shown). In order to avoid this, we decided to grow *C. albicans* biofilms in YPD, but carried out the resazurin assay in MH.

2.5. Biofilm treatment and quantification

Biofilms formed as described above were treated with 100 μl of various antibiotics and antiseptics for 24 h at 37 °C. Contact time for CHX however ranged from 1 to 30 min and reaction was stopped using Dey Engley neutralizing broth (DENB, Lab M, 7 g/l lecithin, 6 g/l sodium thiosulphate, 5 g/l tween 80, 5 g/l tryptone, 2.5 g/l sodium bisulphite, 1 g/l sodium thioglycollate, 0.02 g/l bromocresol purple, 2.5 g/l yeast extract). The supernatants were removed subsequently and the biofilms were rinsed with PS. Conventional plating and resazurin staining were then used to determine the number of culturable and viable biofilm cells, respectively (designed as $CFU_{plating}$ and CFU_{CTB}).

To determine $CFU_{plating}$, 100 μl PS was added to the wells containing the treated biofilms and the MTP was sonicated and vortexed twice (same procedure as described above). The detached cells were quantified by conventional plating. To determine CFU_{CTB}, 100 μl MH was added to the wells and the MTP was sonicated and vortexed twice. Subsequently, 20 μl resazurin was added and the MTP was incubated for 20 h at 37 °C. The fluorescence generated by the reduction of resazurin was measured every 5 min and the time to reach the fluorescence signal of 100,000 and the maximum fluorescence signal were determined. The previous derived calibration curves were then used to calculate the CFU_{CTB}. Finally, $CFU_{plating}$ and CFU_{CTB} were compared for each condition. Statistical analysis was performed with SPSS software using independent sample t-tests. P values <0.01 were considered to be statistically significantly different.

3. Results and discussion

Bacteria growing in biofilms cause persistent infections which are often not cleared with conventional antibiotic treatment alone. New anti-biofilm agents are urgently needed and to this end, good model systems and quantification methods are essential. The non-fluorescent dye resazurin is used to quantify metabolically active cells. In the conventionally used assay, resazurin is diluted in PS and added to biofilms formed and treated in MTP. The fluorescence generated by the reduction of resazurin to its fluorescent reaction product by metabolically active cells is typically measured after 30–120 min. However, the linear range is restricted and the lower limit of quantification is approximately 10^6 CFU per biofilm, which means that the assay cannot discriminate between cell numbers lower than 10^6 CFU per biofilm (Peeters et al., 2007).

In the present study, we optimized this resazurin-based quantification method. Mature *S. aureus*, *P. aeruginosa*, *B. cenocepacia* and *C. albicans* biofilms were formed in MTP. Subsequently, these biofilms were disrupted, detached and collected. Serial dilutions were made and wells of a sterile MTP were filled with these diluted cell suspensions together with fresh MH growth medium and resazurin. The fluorescence generated by the reduction of resazurin was followed over time. The time to reach an absolute fluorescence value of 100,000 and the time to reach the maximum fluorescence were determined for every dilution. The time to reach these fluorescence signals was plotted against the number of CFU present in every dilution, obtained by conventional plating, leading to the calibration curves shown in Fig. 1.

The linear range increased from 10^6–10^8CFU per biofilm as described in Peeters et al. (2007) to approximately 10^3–10^8 CFU per biofilm. This results in a substantially decreased lower limit of