ORIGINAL ARTICLE

Development of a novel biofilm classification tool and comparative analysis of result interpretation methodologies for the evaluation of biofilm forming capacity of bacteria using tissue culture plate method

Monia Avdić[1], Nermin Džuzić[1], Osman Hasanić[1], Amel Spahić[2], Lejla Smajlović-Skenderagić[1], Almir Badnjević[1,3], Mirsada Hukić[4,5]

[1]International Burch University, [2]Mistral Technologies, [3]Verlab Ltd., [4]Institute for Biomedical Diagnostics and Research NALAZ, [5]Academy of Sciences and Arts of Bosnia and Herzegovina; Sarajevo, Bosnia and Herzegovina

ABSTRACT

Aim To develop an online biofilm calculation tool (Biofilm Classifier), which calculates the optical density cut off value and accordingly determines the biofilm forming categories for the tested isolates by standardized formulas, as well as to compare the results obtained by Biofilm Classifier to manual calculations and the use of predefined values.

Methods The biofilm forming capacity of tested strains was evaluated using tissue culture plate method in 96 well plates, and optical density (OD) value of the formed biofilm was measured on an ELISA Microplate reader at 595 nm on a total of 551 bacterial isolates from clinical specimen.

Results Comparative analysis indicated that the manual calculation was 100% in accordance with results obtained by the designed software as opposed to the results obtained by use of predefined values for biofilm categorization. When using predefined values compared to manual biofilm categorization for the determination of biofilm positive and biofilm negative strains the specificity was 100%, sensitivity 97.81%, positive predictive value 100%, negative predictive value 96.04% and accuracy 98.57%.

Conclusion Considering obtained results, the use of the designed online calculator would simplify the interpretation of biofilm forming capacity of bacteria using tissue culture plate method.

Key words: biofilm, cut-off tissue culture plate method, optical density

Corresponding author:
Monia Avdić
[1]International Burch University
Francuske revolucije bb, Ilidža,
71000 Sarajevo, Bosnia and Herzegovina
Phone: +387 62 300 622;
Fax: +387 33 944 500;
E-mail: monia.avdic@ibu.edu.ba
ORCID ID: https://orcid.org/0000-0001-5457-6900

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INTRODUCTION

A gold model for most microbiological examinations is the study of bacteria in pure culture, despite the fact that most bacteria in their natural environment tend to live in associations more commonly known as biofilms (1). A biofilm is an accumulation of microorganisms embedded in a polysaccharide matrix and adherent to a solid biologic or non-biologic surface (1). Due to the increased resistance rates of bacteria embedded in this polysaccharide matrix biofilms have elevated resistance rates to antibiotics and disinfectants (2) and hence they represent a major challenge for microbiologists and clinicians (3). Since biofilms have a significant role in the clinical, industrial and natural setting, the interest in their studying has increased drastically (3).

In the past, there have been many attempts to find simple and reliable methods to detect biofilms. The most commonly used phenotypic assays for the evaluation of biofilm forming capacity today include: the tube test method, Congo red agar method, and tissue culture plate method (TCP) (4), while the detection of biofilm forming genes using polymerase chain reaction (PCR) is amongst the most common genotypic methods. The drawback of the tube test method and Congo red agar method is that they are qualitative tests, they provide results that are neither precise nor sensitive enough. PCR provides more accurate results, however it requires the use of equipment and chemicals that many laboratories do not possess. Therefore, the TCP method is commonly used for the phenotypic evaluation of the biofilm forming potential of species of interest (5,6). Since its very first establishment by Christiansen et al. in 1985 (7) as a quantitative assay for the determination of adherence of staphylococci to medical devices till nowadays, TCP method has been subjected to many modifications and adaptations. Subsequently, scientists in other studies used the values obtained by Christiansen (7) as a referee for the determination of biofilm forming categories (7,8). However, the ODc value should be *de novo* calculated for each new experiment considering the minor changes in the experimental conditions (9), yet this is often avoided due to major workload in the laboratory and time efficiency. Accordingly, tube test is often chosen as a qualitative screening test for the evaluation of the biofilm forming potential of bacterial isolates to avoid calculations needed for TCP. Alternatively, TCP is used, however, biofilm forming categories are determined according to Christiansen's calculations (10).

The aim of our work was to develop an online classification tool for the interpretation of results obtained by the TCP method and at the same time compare the results obtained by this tool to other calculation procedures used for the determination of biofilm forming categories.

MATERIAL AND METHODS

Bacterial strains

A total of 551 bacterial strains isolated from clinical specimen were tested. Criteria for inclusion into the study were that the bacteria originated from clinical specimen and that they were isolated in pure culture. The isolated bacterial strains were inoculated in Lauria Bertani (LB) Broth (Liofilchem Bacteriology Products, Roseto/Italy) supplemented with 50% glycerol and kept at -80ºC. These bacterial strains were recovered from glycerol stocks by plating on blood agar base (Liofilchem Bacteriology Products, Roseto/Italy) and incubated overnight at 37 ºC followed by overnight incubation in tryptic soy broth (TSB) (Liofilchem Bacteriology Products, Roseto/Italy) supplemented with 1% glucose.

Tissue culture plate method

The TCP method was performed according to current protocols in microbiology (6), by inoculating each bacterial isolate in 5 mL of trypticase soy broth (TSB) supplemented with 1% glucose, aerobically in plastic tubes followed by an incubation at 37 °C for 24 hours. The overnight cultures were diluted in 1:100 ratio in TSB in 96 well polystyrene plates and incubated for additional 24 hours at 37 °C. Samples were arranged in quadruplets. The first quadruplet of uninoculated wells (only media) was used as a negative control. After incubation the trays were washed to remove all planktonic bacteria followed by crystal violet staining (0.1%); 96% alcohol was used as a solvent for crystal violet. Upon a 10-minute incubation 125 µL of the crystal violet/ethanol solution from each well was transferred to a separate well in an optically clear flat-bottom 96-well plate. The optical

density (OD) value was measured on Microplate reader RT-6100 (Rayto Life and Analytical Sciences, Shenzhen/China) at 595 nm.

Examination of the biofilm forming capacity

The obtained OD of the negative control in quadruplet was used for the calculation of the ODc (three standard deviations above the mean OD of the negative control), which was further used for the determination of biofilm forming categories per standardized formulas for manual calculation of biofilm forming categories – which was used as a reference (Table 1).

Table 1. Comparative analysis of biofilm categorization using three biofilm interpretation methodologies

Interpretation method	No of bacterial strains			
	Nonadherent	Weak	Moderate	Strong
Predefined value use	202	187	94	68
Manual calculation	194	185	105	67
Online classification tool	194	185	105	67

Development and implementation of biofilm classifier

Biofilm Classifier was built in Angular 5, which is an all-encompassing JavaScript framework for building web, desktop, and mobile applications. The whole code was written in Microsoft Visual Studio Code, following the latest updates for faster and more responsive experience.

The program was tested on a database of a total of 551 clinical bacterial isolates. Results obtained on ELISA Microplate Reader were used as inputs for our web application.

Biofilm classifier tool

Biofilm Classifier available at http://biofilmclass.com is an online classification tool that uses the measured OD values of negative control and according to formula (3STDEV + Mean OD neg contol) calculates the ODc. The ODc was further used to determine the biofilm forming categories according to predefined formulas for all the other test samples (OD ≤ ODc=no biofilm producer; ODc <OD≤2xODc=weak biofilm producer; 2xODc <OD≤4xODc=moderate biofilm producer; 4xODc<OD=strong biofilm producer) (10).

Within the online Classification tool wells of the 96 well plates were grouped into quadruplets (groups of four wells) and labelled with a corresponding letter and number. Since microbiological cultures are usually inoculated vertically in ELISA test, the first two quadruplets (A1-D1 and E1-H1) represent the negative control (containing only microbiological growth medium) whereas other quadruplets represent the tested samples. A number of tested samples (quadruplets) is required as the initial input and ranges from 2 to 24. Subsequently the obtained OD values from the TCP assay are manually entered and the ODc as well as the biofilm forming categories for each sample are determined.

Statistical analysis

The statistical analysis was carried out using MedCalc online program for the determination of a test's sensitivity, specificity, positive and negative predictive value and accuracy.

RESULTS

Comparative analysis of biofilm result interpretation methods

Results of the comparative analysis indicated that the manual calculation was 100% in accordance to the results obtained by the designed software, e. g. online classification tool as opposed to the results obtained by the use of predefined values for the biofilm categorization (Table 1).

The difference in biofilm categorization using predefined values and manual/online classification was less emphasized when the strains were clustered into biofilm positive (weak, moderate and strong) and biofilm negative groups (Table 2). Namely, a total of 8 strains were miss-categorized as biofilm positive using the predefined values for biofilm categorization, which accounts for 1.45% from 551 tested bacterial strains.

Table 2. Comparative analysis of the three tested biofilm interpretation methodologies

Interpretation method	No (%) of bacterial strains	
	Online classification tool/ manual calculations	Predefined value use
Biofilm positive (weak, moderate and strong biofilm forming strains)	357 (64.79)	349 (63.34)
Biofilm negative (non-adherent strains)	194 (35.21)	202 (36.66)

Miscategorizations were more emphasized when biofilm positive strains were grouped into three categories (weak, moderate, strong). In this case the difference in categorization occurred in the total number of 2 weak biofilm forming strains, 11

moderate biofilm forming strains and one strong biofilm forming strain (Table 1). This in total yielded a difference in biofilm categorization of 22 strains (14 biofilm positive and 8 biofilm negative) or 4% from the tested 551 bacterial strains using predefined values for biofilm categorization compared to manual calculation as a reference.

Accordingly, the sensitivity and specificity as well as accuracy of the online classification tool compared to manual calculations as a referee was 100% according to statistical analysis.

On the contrary, when using predefined values for biofilm categorization compared to manual biofilm categorization for the determination of biofilm positive and biofilm negative strains - the sensitivity is 97.81%, specificity 100%, positive predictive value 100%, negative predictive value 96.04% and accuracy 98.57%.

The results obtained by comparing manual calculation testing methodologies as a reference using predefined values for each category (to non-adherent stains) for categorization of moderate biofilm forming strains showed the lowest sensitivity, negative predictive value and accuracy, however, it still was over 90% in all cases. The accuracy for weak and moderate biofilm forming categories using predefined values was over 99% (Table 3).

Table 3. Comparison of the use of predefined values for biofilm category determination to manual calculations using ODc as a reference for each biofilm forming category

	Biofilm forming category		
Results of comparison (%)	**Weak**	**Moderate**	**Strong**
Sensitivity	100	90.52	100
Specificity	98.98	100	99.49
Positive predictive value	98.93	100	98.53
Negative predictive value	100	94.63	100
Accuracy	99.48	96.45	99.62

ODc, three standard deviations above the mean optical density of the negative control

DISCUSSION

Associations of microorganisms attached to surfaces, more commonly known as biofilms, have received significant attention over the past years due to the role they play in the industrial, natural and clinical setting (1,5). Many biofilm research methodologies have been proposed up to now, genetic and phenotypic (8,10,11). However, due to expenses, the use of phenotypic test is more common for the determination of the biofilm forming capacity of tested microbial strains in laboratory conditions. In the realm of phenotypic testing the use of the tissue culture plate method is considered as a reference method (5). Regardless of that fact, a referent result interpretation methodology for the TCP method is still not defined and established (9).

For the determination of the cut-off value for the TCP method up to now many result interpretation methodologies are encountered and they include: the use of predefined values established by Christiansen in 1985 (8), cut-off calculations based on three standard deviations above the mean OD of the negative control or a biofilm negative strain, considering a strain positive if it is twice the OD of the negative control and some even use the value obtained by the positive control as the starting point (1,2,9, 12-21).

Interpretation procedure that is considered most reliable is the calculation of the cut off based on three standard deviations above the mean OD of the negative control for each 96 well plate separately and subsequent biofilm categorization based upon the use of standardized formulas (10). This falls under the definition of an endpoint titre that is defined as the reciprocal of the highest analyte dilution that gives a reading above the cut off values (22). The fact that no standardized procedure is established for end point titres where there is no positive standard for immunoassay results opens the possibility of use of different calculation procedures as seen in the case of biofilm classification (9,22). In the field of healthcare many automated systems have been developed for the purpose of classification or prediction (23-28).

Since biofilms represent a new frontier in microbiology and are intensively studied, a need to establish result interpretation guidelines for the most commonly used TCP method that would at the same time enable the comparison of data obtained from previous studies would be a milestone in the future studies of this phenomena. Hence the main aim of our study was to develop an online classification tool, available to all, that would aid scientists to determine the biofilm forming capacity of the tested bacterial strains using a common result interpretation method that is not time consuming and is considered most reliable according to previous work (9,22). Besides that, considering the number of studies that employed

the use of predefined values for biofilm classification established by Christiansen (7) in our work we gave a comparison of three evaluation procedures: manual calculation, online classification tool and use of predefined values, and determined their sensitivity, specificity and accuracy.

The results of our study showed that the categorization using the developed online biofilm classification tool were 100% in accordance to the results obtained by the use of manual calculations. The sensitivity and specificity of this calculator both were 100%.

Our study showed that the comparison of the use of predefined values for biofilm categorization to the use of manual calculations missed to classify 1.45% of the strains that produced biofilms. The specificity and positive predictive value were as high as 100%, while the sensitivity was lower at 97.81%, negative predictive value 96.04% and accuracy 98.57%. These parameters were high considering that test results above 95% are considered reliable for the use in laboratory conditions.

When comparing manual calculation methodologies as a referee to the use of predefined values for each category (to non-adherent stains) the results of this study for the categorization of moderate biofilm forming strains showed the lowest sensitivity, negative predictive value and accuracy, however, it was still over 90% in all of the cases. The accuracy for weak and moderate biofilm forming categories using predefined values was over 99%. This suggests that the use of predefined values for the biofilm categorization could potentially be used for the determination of biofilm forming categories since it has a sensitivity, specificity and accuracy over 90%. However, the results can vary to a certain extent and the use of an online tool for exact calculations would aid in the establishment of a referee as a result interpretation methodology for all biofilm classifications using the tissue culture plate method. At the same time using such a referee would allow the comparison of results obtained from different studies and in doing so contribute to the further development of biofilm studies, which represent a new frontier in microbiology today.

The limitation of our study was that only two biofilm result interpretation methodologies were compared while multiple have been used up to now. Hence in our future work we aim to compare the results of all used result interpretation procedures found in literature to enable comparisons of the obtained results.

Due to the fact that biofilms represent a new frontier in microbiology, the establishment of referent testing procedures available and applicable to all in the realm of phenotypic biofilm testing procedures is mandatory for future development of the field.

In our study we developed the first online classification tool for biofilm categorization using TCP method and in doing so set a milestone for establishing a unique result interpretation method for future biofilm studies where the determination of biofilm forming categories for tested samples is a necessity.

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TRANSPARENCY DECLARATION

Competing interests: None to declare.

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