A Novel Spectrophotometric Assay for the Determination of Biofilm Forming Capacity of Causative Agents of Urinary Tract Infections

Monia Avdić Ibrišimović,
Department of Genetics and Bioengineering,
International Burch University,
Sarajevo, Bosnia and Herzegovina

Mirza Ibrišimović,
Sarajevo Medical School, University Sarajevo
School of Science and Technology (SSST),
Sarajevo, Bosnia and Herzegovina

Nadira Ibrišimović Mehmedinović,
Department of Chemistry,
Faculty of Science and Mathematics,
University of Tuzla,
Tuzla, Bosnia and Herzegovina

Mirsada Hukić,
Institute for Biomedical Diagnostics and Research
NALAZ,
Sarajevo, Bosnia and Herzegovina

Abstract— According to many studies today the biofilms play a crucial role in the clinical setting and are the cause of many difficult to treat and reoccuring infections. Recently their role in urinary tract infections (UTIs) is becoming very significant as they are known to cause relapses and reoccuring infections especially in patients with indwelling medical devices. Up to today many biofilm testing methodologies have been suggested, however, all of them have certain drawbacks and routine testing of the biofilm forming capacity of causative agents of infection is not conducted. In our study we developed a novel spectrophotometric assay for the evaluation of the biofilm forming capacity of causative agents of UTIs and tested it on 120 urine samples isolated from two medical centars in Mostar, Bosnia and Herzegovina. The results of the novel spectrophotometric assay were then compared to the results obtained by the tissue culture plate method which was used as a referee. Based on statistical data the sensitivity and specificity of the novel spectrophotometric assay was evaluated to be 95% which is adequate for the use in standard clinical microbiology laboratories for the determination of the biofilm forming capacity of causative agents of UTI.

Keywords— Biofilms; Bacteriology; Spectrophotometry; Urine; UTI.

I. INTRODUCTION

The approach of classical bacteriology is based on "the pure culture technique" which implies the culturing of planktonic or "free floating" bacteria, from various samples, on adequate enriched solid or liquid media and their subsequent analysis upon incubation (biochemical characteristics and antibiotic susceptibility testing) [1, 2]. Today, this is still considered the „Gold Model", for most microbiological examinations in the clinical setting. However, in natural environments, like host tissues and on surfaces, most bacteria, exist in complex associations more commonly known as biofilms [3, 4, 5]. A biofilm is an accumulation of microorganisms embedded in a polysaccharide matrix and adherent to solid biologic or non-biologic surface" [4]. Considering the gold standard, the study of only planktonic pure cultures and by this methodology obtained results are

highly questionable as well as the results acquired by subsequent antibiotic testing [6, 7, 8, 9].

Because of the significant role that biofilms have in the clinical, industrial and natural setting, the interest in their studying has increased drastically [10]. Today many infections are known to be biofilm associated [11, 12, 13, 14]. There have been many attempts to find simple and reliable methods to diagnose biofilm-associated infections some of which include the tube method, congo red agar method, bioluminescent assay, piezoelectric sensors, fluorescent microscopic examination as well as the tissue culture plate method and PCR [15]. The most commonly used assays include the congo red agar method and tube test method, which are qualitative tests with low sensitivity and specificity, and the tissue culture plate method and PCR, that require the use of costly chemicals as well as sophisticated equipment that most small laboratories do not possess [16, 17, 18, 19, 20, 21, 22].

On the other hand, despite the prevalence of biofilm associated infections, routine testing of the biofilm forming capacity of the causative agents of various infections, including UTIs, is not conducted. This is alarming, especially in the treatment of UTIs, which are the most common infections. Their treatment implies the use of antibiotics, in some cases even in empirical therapy that as a consequence may enhance the biofilm forming capacity of these strains [23, 24, 25, 26].

Therefore, the aim of our study was the development of a sensitive, specific and cost effective spectrophotometric assay for the evaluation of biofilm forming capacity of strains isolated from urine of patients suffering from UTI - by optimizing the evaluation of the extent of formed biofilm from qualitative tests.

II. MATERIALS AND METHODS

A. Sample Collection and Preparation

A total of 120 bacterial isolates, obtained from urine and catheter specimen from hospitalized patients, as well as

outpatients, were collected in two microbiology laboratories in Mostar, Bosnia and Herzegovina: Laboratory for microbiology and parasitology "Dr. Safet Mujić" – Cantonal Hospital (secondary care center) and Laboratory for microbiology and molecular diagnostics University Clinical Hospital (tertiary medical center).

The criteria for inclusion into the study was that the sample had only one causative agent of UTI and that only one of three successive urine samples from the same patient was taken into consideration. The collected urine samples were tested for their physical, biochemical and microscopic characteristics and only the samples that implied possible UTI, according to all three tested criteria, were further analyzed. Species identification was done according to biochemical tests. The results obtained by the tissue culture plate method were used as a referee for the determination of the biofilm forming capacity of the tested strains.

B. Tissue Culture Plate Method (TCP)

Each bacterial isolate was inoculated in 5 ml of trypticase soy broth (TSB) supplemented with 1% glucose, aerobically in plastic tubes and incubated at 37°C for 24 hours. The overnight cultures were diluted 1:100 in a desired medium in 96 well polystyrene plates (in quadruplets) and incubated for an additional 24 hours at 37°C. The first four wells were not inoculated and they were used as a negative control. After incubation the trays were emptied, submerged in water trays and emptied again to remove all planktonic bacteria. After washing, 125 µl of 0.1% crystal violet solution was added to each well and incubated for 10 minutes at room temperature. The crystal violet solution was removed from the trays and the trays were washed in a water tray successively. To each well, 200 µl of 96% alcohol, as solvent, was added and incubated for 15 minutes at room temperature to allow the stain to solubilize upon which 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) was measured on an ELISA plate reader at 600 nm. The absorbance values from these replicates are sufficient to determine an average and standard deviation for each strain or species, and thus provide a measure of the extent of biofilm formation. Based on this all the tested bacterial strains were classified into one of four possible categories: non-adherent, weak, moderate and strong biofilm formers. Since all samples were analyzed in quadruplets the obtained average absorbance value was used for the biofilm forming capacity determination.

The cut-off OD for the determination of biofilm formation was calculated per standardized formula, as three standard deviations above the mean OD of the negative control. Biofilm forming capacity of the tested strains was further calculated per formulas given in table 1. The obtained average value was used as a positive control as advised by standardized procedures.

TABLE 1. FORMULAS FOR CLASSIFICATION OF BIOFILM FORMING CAPACITY FOR TCP METHOD

Formula	Biofilm Formation
OD < ODc	Non adherent
ODc < OD ≤ 2OD	Weak
2ODc < OD ≤ 4OD	Moderate
4ODc < OD	Strong

C. Spectrophotometric Assay for Biofilm Detection

The optimal absorbance for the used crystal violet stain was determined by measuring the spectra for the used stain on a spectrophotometer. Consequently all further measurements were carried out at this wavelength. Each bacterial isolate was inoculated in 5 ml of TSB supplemented with 1% glucose, aerobically in plastic tubes and incubated at 37°C for 48 hours. The content of the tubes was emptied upon incubation and washed three times with PBS to remove planktonic bacteria. The tubes were subsequently stained with 1 ml of crystal violet solution and incubated for 15 minutes. The stain was removed by washing in water. The tubes were left in an inverted position to dry, upon which 5 ml of 96% ethanol was added to each tube and incubated for an additional 15 min. 1 ml of the ethanol/crystal violet solution was transferred into plastic cuvettes and absorbance was measured at 595 nm. Uninoculated tubes were used as a negative control, while strains that displayed strong biofilm formation according to the TCP were used as a positive control.

D. Statistical Analysis

The results obtained by the tissue culture plate method were compared to the results obtained by the spectrophotometric assay and specificity, sensitivity, positive and negative predictive value as well as positive likehood and negative likehood ratio were determined.

III. RESULTS

A. The Most Prevalent Urine Bacterial Isolates and Biofilm Formers Were Enterococcus spp. and Escherichia coli

From the obtained 120 urine and catheter specimen (from two medical centers in Mostar, Bosnia and Herzegovina), among the isolated bacteria *Escherichia coli* prevailed (52.5%), followed by *Enterococcus spp.* (17.5%), *Staphylococcus spp.* (9.16%) and various *Enterobacteriaceae* (16.65%): Klebsiella pneumoniae (9,16%), Proteus mirabilis (33%), *Pseudomonas aeruginosa* (4.16%), *Enterobacter cloacae* (2.5%), *Providentia spp.* (0.83%) and *Citrobacter diversus* (0.83%) (Table 2).

According to the TCP method 21 strains were identified as non-adherent (average absorbance values (Abs) below 0.117), 38 as weak biofilm formers (average absorbance value between 0.117 and 0.234), 27 as moderate biofilm formers (Abs: 0.234 – 0.469), and 34 as strong biofilm formers (Abs: above 0.469) (Table 3).

The cut-off OD for the determination of biofilm formation was determined according to the negative control which had the average absorbance value at OD595 of 0.0626, while the ODc was calculated according to the given formula to be 0.117. These results were compared to the results obtained by the spectrophotometric assay.

TABLE 2. ISOLATED BACTERIAL SPECIES IN TESTED 120 URINE AND CATHETER SPECMENS

Genus/Species	H1	H2	Total
Escherichia coli	11	52	63 (52.5%)
Enetrococcus spp.	2	19	21 (17.5%)
Staphylococcus spp.	1	10	11 (9.16%)
Klebsiella pneumoniae	5	6	11 (9.16%)
Pseudomonas aeruginosa	4	1	5 (4.16%)
Proteus mirabilis	0	4	4 (3.33%)
Eneterobacter cloacae	2	1	3 (2.5%)
Providentia spp.	0	1	1 (0.83%)
Citrobacter diversus	0	1	1 (0.83%)
Total	25	95	120
H1: Cantonal Hospital ”Dr. Safet Mujić” H2: University Clinical Hospital Mostar			

TABLE 3. BIOFILM FORMATION RESULTS ACCORDING TO TISSUE CULTURE PLATE (TCP) METHOD

Biofilm classification	OD range	Number of bacterial isolates	
Non adherent	< 0.117	21	(17%)
Weak	0.117 – 0.234	38	(32%)
Moderate	0.234 – 0.469	27	(23%)
Strong	< 0.469	34	(28%)
Total		120	(100%)

B. A Novel Spectrophotometric Assay Enabled Sensitive and Specific Determination of Biofilm Forming Capacity of Tested Bacterial Isolates

According to the results obtained by the spetrophotometric assay, from 120 analyzed urine and catheter specimens, 21 isolates were categorized as non- adherent, 41 as weak biofilm formers, 30 as moderate biofilm formers and 28 as strong biofilm formers (Table 4).

The cut-off OD for the spectrophotomteric determination of biofilm formation was calculated as three standard deviations above the mean OD of the negative control.

Classification of bacterial adherence was calculated as per given formulas and bacterial adherence was determined as one of four possible categories: non- adherent, weak, moderate and strong biofilm formation (Table 1).

TABLE 4. RESULTS OBTAINED BY THE SPECTROPHOTOMETRIC ASSAY

Biofilm classification	Calculated OD range	Number of bacterial isolates
Non adherent	< 0.077	21 (17.5%)
Weak	0.077 – 0.154	41 (34.17%)
Moderate	0.154 – 0.308	30 (25%)
Strong	> 0.308	28 (23.33%)
Total		120 (100%)

According to the results of the TCP method 34 strains were identified as strong biofilm formers, 27 as moderate biofilm formers, 38 as weak biofilm formers and 21 as non-adherent. Similar results were obtained by the spectrophotmetric assay according to which, among the isolated strains, 28 strains were identified as strong biofilm formers, 30 as moderate biofilm formers, 41 as weak biofilm formers and 21 as non-adherent (Table 5).

TABLE 5. COMPARISON OF SPECTROPHOTOMETRIC ASSAY AND TISSUE CULTURE PLATE (TCP) METHOD

Biofilm Classification	Method		STDEV
	TCP	Spectrophotometric	
Strong	34	28	4.242
	28.33%	23.33%	
Moderate	27	30	2.121
	22.50%	25%	
Weak	38	41	2.121
	31.66%	34.17%	
Non adherent	21	21	0
	17.50%	17.50%	
TOTAL	120	120	

The spectrophotometric assay recognized 41 strains as weak biofilm formers. However, according to the TCP methodology 38 strains were identified as weak biofilm formers.

The strains that were identified as weak biofilm formers according to the spectrophotometric assay and as moderate biofilm formers in the TCP assay included 1 *E. coli* strain, 1 Enterococcus spp. strain and 1 strain of *Enterobacter cloacae* (Table 6).

TABLE 6. BIOFILM FORMING CAPACITY OF THE IDENTIFIED 120 STRAINS, ISOLATED FROM URINE AND CATHETER SPECIMEN, ACCORDING TO THE SPECTROPHOTOMETRIC ASSAY

Genus/species	Biofilm Classification				
	Non-adherent	Weak	Moderate	Strong	Total
Escherichia coli	13 (20.63%)	29 (46.03%)	16 (25.39%)	5 (7.93%)	63 (100%)
Enetrococcus spp.	3 (14.28%)	3 (14.28%)	6 (28.57%)	9 (42.85%)	21 (100%)
Staphylococcus spp.	2 (18.18%)	6 (54.54%)	0 (0%)	3 (27.27%)	11 (100%)
Klebsiella pneumonia	1 (9.09%)	2 (18.18%)	4 (36.36%)	4 (36.36%)	11 (100%)
Pseudomonas aeruginosa	0 (0%)	0 (0%)	2 (40%)	3 (60%)	5 (100%)
Proteus mirabilis	1 (25%)	0 (0%)	1 (25%)	2 (50%)	4 (100%)
Eneterobacter cloacae	0 (0%)	1 (33.33%)	1 (33.33%)	1 (33.33%)	3 (100%)
Providentia spp.	1 (100%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)
Citrobacter diversus	0 (0%)	0 (0%)	0 (0%)	1 (0%)	1 (100%)
Total	21	41	30	28	120 (100%)

According to the spectrophotometric assay 30 strains were identified as moderate biofilm formers (while the TCP recognized only 27) and 28 strong biofilm formers (while TCP recognized 32).

C. Statistical Analaysis of the Spectrophotometric Assay and Tissue Culture Plate Method for Precise Estimation of Biofilm Forming Capacity

The sensitivity of the spectrophotometric assay was calculated to be 95.08%, while the specificity was estimated to be 95.16%. Positive likehood ratio had a value of 19.65 and negative likehood ratio had a value of 0.05. Positive predictive value was calculated to be 95.08% while negative predictive value was calculated to be 95.16% (Table 7).

TABLE 7. STATISTICAL ANALYSIS OF THE SPECTROPHOTOMETRIC ASSAY AND TISSUE CULTURE PLATE METHOD FOR THE ESTIMATION OF BIOFILM FORMATION

Statistic	Formula	Value	95% CI
Sensitivity	$\dfrac{a}{a+b}$	95.08%	86.29% to 98.97%
Specificity	$\dfrac{d}{c+d}$	95.16 %	86.50% to 98.99%
Positive Likelihood Ratio	$\dfrac{Sensitivity}{100-Specificity}$	19.65	6.51 to 59.35
Negative Likelihood Ratio	$\dfrac{100-Sensitivity}{Specificity}$	0.05	0.02 to 0.16
Disease prevalence	$\dfrac{a+b}{a+b+c+d}$	49.59% (*)	40.46% to 58.75%
Positive Predictive Value	$\dfrac{a}{a+c}$	95.08% (*)	86.29% to 98.97%
Negative Predictive Value	$\dfrac{d}{b+d}$	95.16 % (*)	86.50% to 98.99%

IV. DISCUSSION

Biofilms were recognized as early as the 17th century, however it was not until the 1970's that an accent was given to the significant role they play in the clinical setting [27, 28, 29, 30]. With the discovery of biofilm based infection, the classical approaches which were used in microbiology for decades became questionable for the study of these complex associations of microorganisms [31, 32, 33, 34]. Recently biofilms have become a major concern in urinary tract infections where they are known to cause relapses and persistent infections and a special aspect of these infections is in patients that are catheterized [35, 36, 37, 38]. Up to today many biofilm testing methodologies have been suggested, however all of them have certain drawbacks and are therefore not routinely used in clinical microbiology laboratories. Qualitative tests like the tube test and congo red agar test lack sensitivity and specificity while other tests like the tissue culture plate test and polymerase chain reaction (PCR) require the use of sophisticated equipment. Considering this the need to develop a sensitive and specific test that is at the same time cost effective for the determination of biofilm forming capacity of causative agents of infection is a priority as a milestone in the development of a novel approach to the study of these complex associations of microorganisms that are the cause of many infections.

Since routine testing of the biofilm forming capacity of causative agents of UTI is not regularly performed, in our study using the tissue culture plate method as a referee, all the 120 strains were tested for their biofilm forming capacity. The tissue culture plate method was chosen as a referee since it was used in a number of different studies, where clinical samples were tested, and it proved to give valid results [16, 17, 18, 19, 20, 21, 22]. Further confirmation of the validity of this quantitative test was done in protocols suggested by genetic approaches to biofilm study. There it is stated that TCP is a valid phenotypic test for the determination of biofilm forming capacity of analyzed bacterial strains [10].

Since the TCP method results can only be read on an ELISA plate reader that has the adequate filters (crystal violet: 570-600nm, safranin 490nm), this method is often avoided in small clinical laboratories that do not possess the adequate equipment to carry out this assay. Therefore, despite the validity of the mentioned quantitative assay as a phenotypic test for the determination of biofilm forming capacity of various causative agents of infection, it is often avoided. In the search for a novel approach for the detection of causative agents of biofilm based urinary tract infections, the major drawback of the qualitative tube test was the evaluation of the extent of the formed biofilm. By optimizing the procedure for the measurement of the extent of formed biofilm, which is proportional to the quantity of the crystal violet stain that remains on the tubes upon washing, a novel quantitative approach that allows the use of only a spectrophotometer was developed.

From all the studies done on biofilm based infections up to now, it is clear that we are faced with a new frontier in microbiology which questions the entire classical approach to bacteriology as a whole. Namely, bacterial biofilms represent a natural state of these organisms and the results obtained by the in vitro study of pure cultures as a golden model, without the prior testing of the biofilm forming capacity of the causative agents of infection, are becoming questionable. Considering this, the necessity of establishing routine testing of the biofilm forming capacity of causative agents of UTI and the development of sensitive, specific and cost effective assays affordable to all clinical microbiology laboratories is just a milestone in the development of novel approaches to the study of biofilm based infections as a new frontier in microbiology.

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