BIOTIMER ASSAY FOR COUNTING BACTERIAL BIOFILM

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Abstract: A growing body of evidences shows that bacterial biofilm lifestyle is comparatively more common than the planktonic one and that biofilm plays a crucial role in human health. The enumeration of the actual number of bacteria in biofilm is still a great challenge for microbiologist. The standardized method of colony forming unit (CFU) count is not reliable to quantify bacteria in biofilm. In the absence of a validated method to count bacteria in biofilm, BioTimer Assay (BTA) is presented here. BTA allows to count bacterial biofilm adherent on surfaces and employs an appropriate reagent containing an indicator able to switch as a consequence of bacterial metabolism in biofilm. The time required for indicator switch, induced by microbial metabolism, is correlated to the initial number of bacteria (N0) through a genus-specific correlation line described by the following equation $t* = log(1 + a/N0)/k$ where k is growth rate and a is a function of the metabolic product responsible for the reagent switching. Moreover, BTA does not require any manipulation of samples and has been applied to count bacteria in biofilm adherent to several polymers, to verify microbiological quality of foods and to detect the antibiotic susceptibility of biofilm. BTA, providing a reliable, sensitive, rapid and easy-to perform method, could be considered a useful tool in counting bacteria in biofilm also adherent on nano-structured particles to be in vivo administered.

1 INTRODUCTION

Biofilms are multicellular communities held together by a self-produced extracellular matrix. Bacteria possess the ability to form biofilms in response to many factors, which may include cellular recognition of specific or non-specific attachment sites on a surface, nutritional cues, or in some cases, by exposure of planktonic cells to sub-inhibitory concentrations of antibiotics.

Bacterial biofilm lifestyle is comparatively more common than the planktonic one and it has been shown that biofilm plays a crucial role in human health (Brady et al., 2008; Bryers, 2008). A fundamental prerequisite in studying, controlling and/or counteracting biofilm formation and development is the possibility of quantifying the actual number of bacteria involved. Bacterial counts have deep implications in microbiological diagnosis and therapeutic treatments (Bryers, 2008), in water and food quality analysis (Ramalho et al., 2001; Lee et al., 2007; Rueckert et al., 2005), in environmental applications and consumers' safety. The standard method used to evaluate the number of bacteria, based on determination of the Colony Forming Units (CFUs), can be considered fully appropriate only when bacterial cells are in planktonic lifestyle but is unreliable to count bacteria in biofilm (Berlutti et al., 2003). Even if different analytical strategies have been attempted to enumerate bacteria in biofilm, the detection of the actual number of bacteria adherent in biofilm is still a great challenge for microbiologists.

Direct microscopic observation of bacteria adherent to surfaces through the use of optical, scanning electron, and transmission electron microscopes have been employed (Verheyen et al. 1993; John et al. 2001; Vacheethasanee et al. 2000), but these methods present difficulties in assessing cell viability and in counting bacteria in aggregated or biofilm lifestyle. However, a microscopic method based on the fluorescent stain LIVE/DEAD Viability Kit (Bac-Light) allows to discriminate live from dead cells (Boulos et al.1999; Hope et al. 2002). Gottenbos et al. (2002) using the LIVE/DEAD Viability Kit together with laser scanning confocal microscopy and image analysis software, were able to reliably count adherent bacteria. There are limitations in the use of these techniques due to equipment complexity and to the need to observe and count bacteria in several microscopic fields in order to transform qualitative into quantitative data. Moreover, Atomic Force Microscopy has permitted to observe that the defects of biomaterials are related to the different adhesion efficiency of bacteria as well as to different biofilm formation, thus indicating the limitations of these analytical assays in quantitative detection of bacteria. Other methods suggest to detach bacteria by vortex or sonication and successively to count detached bacteria by CFU technique (Ceri et al.,1999; Sandoe et al., 2006). These procedures present disadvantages as they may not detach all bacteria or may alter bacterial viability leading to erroneous counts. Some methods count bacteria in biofilm through indirect assays based on staining the firmly adherent cells (Christensen et al. 1985) or on spectrophotometrically measuring the dye eluted from stained bacteria (Merritt et al. 1998). These methods, too, are subject to uncertainties: the bacteria could be nonhomogeneously stained, or the dye also could be adsorbed to the abiotic materials.

Other microbiologic assays that count bacteria on the basis of cell metabolism appear more actual to count bacteria adherent to hard surfaces. At present, two methods are available. The first one (Total Microbe Hunter) is marketed by Crescent Chemical Company, Inc. (New York, NY), and it is based on the biologic reduction of the redox indicator 2,3,5 triphenyl tetrazolium chloride by metabolically active microorganisms (Bochner et al 1977). The other (BacT/Alert) is marketed by BioMerieux-Organon Teknika B.V. (Marci lEtoile, France) and is based on the colorimetric detection of CO2 produced by viable microorganisms (Thorpe et al. 1990). However, these methods are semi-quantitative. Moreover, particularly relevant is the count of bacteria adherent in biofilm on the catheters or medical devices (Bestul and Vandenbussche, 2005; Falagas et al., 2007). Usually, antibiotic treatment of catheter-related infections is based on antibiotic susceptibility tests performed on planktonic counterpart of the clinical isolates instead of on biofilm. It is well known that microorganisms organized in biofilm exhibit higher levels of antibiotic resistance than in planktonic form, so that a great part of therapeutic regimens based on susceptibility of planktonic forms fails to eradicate biofilm infections (Carratal, 2002; Pascual et al.,1993). The Calgary Biofilm Device, the most popular method (Ceri et al., 1999), determines the minimal biofilm eradication concentration (MBEC) as the concentration of antibiotic killing 100% of bacteria in biofilm. Unfortunately, this method dont detect the actual number of bacteria in biofilm used as inoculum in MBEC tests. As inoculum size influences the results of susceptibility tests (Egervarn et al., 2007), MBEC values determined using the above mentioned methods, could be mistaken. Taken together the data reported, it is imperative to setup a reliable method to detect the number of bacteria in biofilm.

Here, we describe a novel method named BioTimer Assay (BTA) that allows easily to count bacteria in biofilm without any manipulation of samples. BTA is an indirect method counting bacteria in biofilm by detecting the

time required for an indicator switch induced by a metabolic product specific for each bacterial genus.

2 BIOTIMER ASSAY

BioTimer assay (BTA) utilizes a specific reagent added with 25 mg/L phenol red (BT-PR reagent) to count fermenting bacteria (Berlutti et al 2003; Pantanella et al 2008). After sterilization at 121 C for 15 min, pH is checked and adjusted at 7.2±0.1. The final reagent appears clear and red. BTA measures microbial metabolism: the time required for colour switch of phenol red indicator in BT-PR reagent (red-to-yellow) (Fig. 1), due to bacterial metabolism, is correlated to initial bacterial concentration. Therefore, the time required for colour switch determines the number of bacteria present in a sample at time = 0 through a correlation line. To draw the correlation line specific for *Staphylococcus aureus, Escherichia coli, Streptococcus sobrinus, Enterococcus faecalis and Streptococcus oralis*, 0.2 ml of overnight specific cultures are added to 1.8 ml of BT-PR reagent. Serial two-fold dilutions in 1 ml of BT-PR reagent are performed in 24-well plates (BD, Italy) and simultaneously counted using colony forming unit (CFU) method. Incubation is performed at 37 C without shaking. The colour of the inoculated BT-PR reagent is checked at regular time intervals. For each two-fold dilution, the time required for colour switch of BT-PR reagent is recorded and plotted versus the log10 of CFUs (Figure 2). As the correlation lines link the time for colour switch of BT-PR reagent and the CFUs of planktonic bacteria, the number of bacteria in biofilm can be defined as planktonic-equivalent CFUs (PE-CFUs). A different reagent, containing 10 mg/L of resazurin, named BioTimer-resazurin (BT-RZ), is employed to count non-fermenting bacteria including *Pseudomonas aeruginosa*. After sterilization at 121 C for 15 min, pH is checked and adjusted at 7.0±0.1. The final reagent appears clear and blue. The time required for colour switch of resazurin indicator in BT-RZ reagent (blue to pink) (Figure 3), due to bacterial metabolism, is related to initial bacterial concentration through a specific correlation line (Figure 4). All correlation lines are obtained by linear regression analysis, and linear correlation coefficients are calculated from the equation:

$$r = (n\sum xy - \sum x \sum y)/(sqrt((n\sum x^2 - (\sum x)^2)(n\sum y^2 - (\sum y)^2))). \quad (1)$$

3 BIOTIMER APPLICATIONS

It is important to underline that the counts of bacteria in biofilm, through BTA, do not require any manipulation of the samples, and this characteristic represents an important advantage of BTA with respect to other methods. However, in the absence of a validated reference method, the number of bacteria in biofilm carried out by BTA cannot be compared with those obtained by other methods to enumerate bacteria in biofilm and this deficiency is a disadvantage for our novel method. Notwithstanding that, BTA has been successfully applied to enumerate bacteria in biofilm adherent on polymers, on different foods and recently, to detect the susceptibility of biofilm to antibiotics as well as the microbiological quality of nano-particles to be in vivo administered.

3.1 BioTimer Assay in counting bacterial biofilm adherent on poly(HEMA)-based hydrogels

The first application has been carried out to detect bacterial adhesion to different poly(HEMA)-based hydrogels for dental restorative procedures (Berlutti et al 2003). In conditions that mimic those present in the oral cavity, *S. sobrinus and S. oralis* adhesion on different polymers has been detected. The enumeration by BTA of these two bacterial species adherent in biofilm to polymers has demonstrated that the physico-chemical characteristics of poly(HEMA)-based hydrogels are the major factors promoting bacterial adhesion, which increased with increasing water content in the swollen polymers, reaching maximal values on the cationic polymers (Figure 5). Therefore, BTA is a suitable method to actually distinguish polymers exerting different influence on bacterial adhesion in biofilm.

3.2 BioTimer Assay in counting *Escherichia coli* in foods

BTA has been also tested in preventing foodborne diseases. The correct procedure to ensure an effective prevention of foodborne diseases consists essentially in microbiological monitoring and enumeration of *E. coli* as indicator of faecal contamination at critical control points along the food producing procedures. A total of 122 samples were analysed using both BTA and Reference method (CFU counts). BTA results showed an overall agreement percentage with Reference method even if the number of *E. coli* detected by BTA is higher than that observed by CFU counts (Berlutti et el 2008). Moreover, the time required to obtain the results of bacterial counts with BTA was 3-fold shorter than that employed with Reference one (Figure 6). BTA method may be considered an useful tool for detection of *E. coli* contamination in foods and it may be successfully employed in risk analysis of foodborne diseases.

3.3 BioTimer Assay in detecting antibiotic susceptibility of bacteria adherent in biofilm

An other set of experiments has been performed to verify the possibility to use BTA in detecting antibiotic susceptibility of bacteria adherent in biofilm. As matter of fact, the medical device-related infections are frequently a consequence of Staphylococcus adherent in biofilm, a lifestyle enhancing bacterial resistance to antibiotics. Antibiotic susceptibility tests are usually performed on planktonic forms of clinical isolates. As already reported, Calgary device has been developed to perform antibiotic susceptibility tests on biofilm (Ceri et al., 1999). However, this method does not count bacterial inoculum. As antibiotic susceptibility is related to bacterial inoculum, the test results could be mistaken. Therefore, BTA has been employed to count bacteria in biofilm and to analyze biofilm antibiotic susceptibility (Pantanella et al 2008). Results confirm that BTA is suitable to count bacteria in biofilm and to quantitatively detect the higher resistance to antibiotics by Staphylococcus biofilm respect to planktonic lifestyle (Figure 7).

3.4 BioTimer Assay in in counting *Streptococcus mutans* adherent in biofilm on nano-structured particles.

A coating of single wall carbon nanotubes (SWCNTs) was used to produce glass beads (GBs) having a nano-structured surface (SWCNT-GBs). Both uncoated- and SWCNT-coated GBs were sterilized by autoclaving at 121C for 15 min. After sterilization, uncoated- and SWCNT-coated GBs were washed three times in sterile distilled water before the sterility assay. SWCNTs were added to BTA specific to count fermenting bacteria. It has been observed that the reagent did not switch, indicating the sterility of GBs and SWCNT-GBs. Moreover, both uncoated- and SWCNT-coated GBs were inoculated with a total of 3.2 ± 0.3*105 *Streptococcus mutants* to quantitatively evaluate bacterial adhesion efficiency on uncoated- and SWCNT-coated GBs by BTA. The results have shown that the number of bacteria adherent on SWCNT-coated GBs (1.2 ± 0.3*105) was higher than that detected on uncoated-GBs (3.2 ± 0.3*104). The AFM images of adherent *Streptococcus mutants* on carbon nanotubes are reported in Figure 8.

4 Conclusions

To our knowledge, BTA is the first method that counts adherent bacteria in biofilm or bacteria contaminating foods without any manipulation of samples. BTA is an easy-to-perform and reliable method to assay also biofilm susceptibility to antibiotics. This method does not require sophisticated apparatus as it only requires to draw a correlation line for each bacterial genus tested. Recently, BTA has been employed to count bacteria in biofilm adherent on nano-structured particles. Preliminary results encourage us to apply BTA on sterility and microbiological quality control of nano-particles to be in vivo administered.

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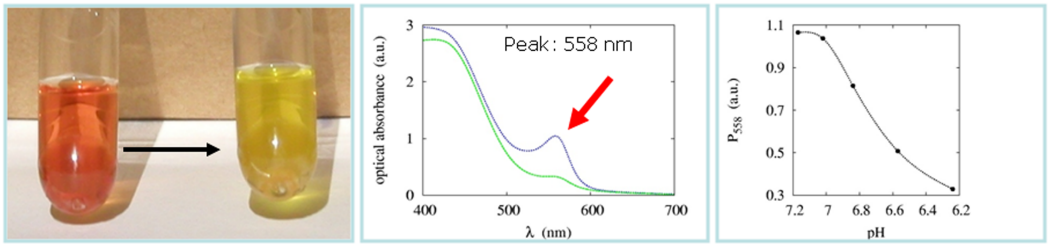
5 FIGURES

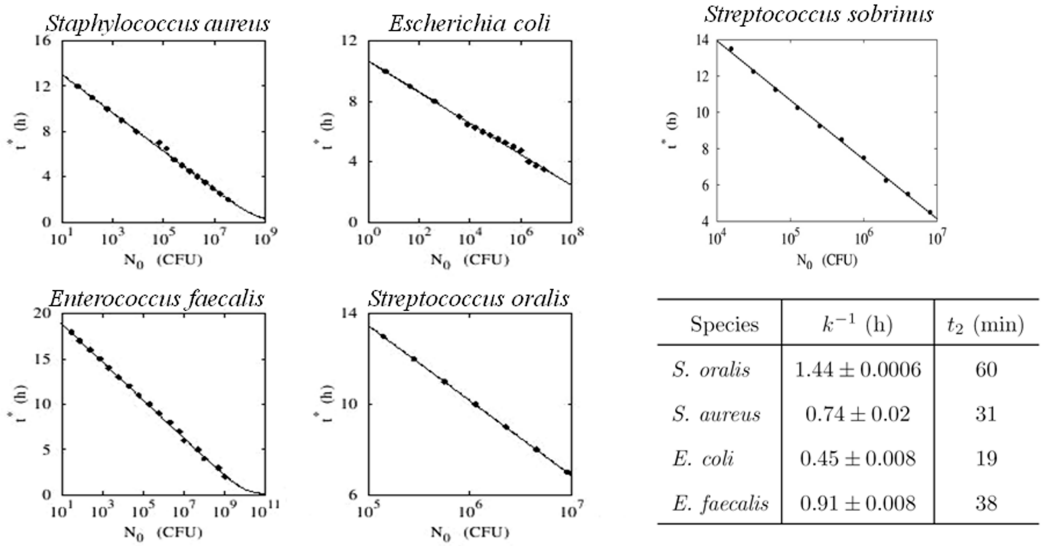


Reagent with phenol red: initial color (left, red) and after switching (right, yellow).

Spectrophotometric detection: initial and switched reagent (blue and green line, respectively)

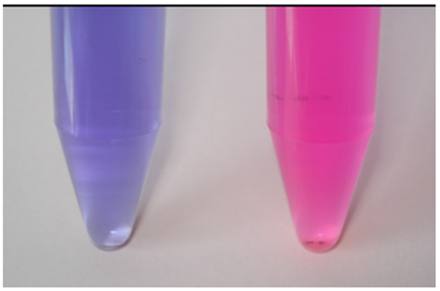
Reagent switch as function of pH (λ 558)

Figure 1: *BioTimer reagent to count fermenting bacteria.* BTA utilizes an original, appropriate reagent containing a colorimetric indicator able to switch as a consequence of the fermenting metabolism of bacteria in planktonic and biofilm lifestyle

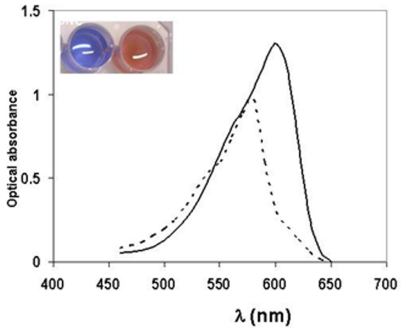


Species	k^{-1} (h)	t_2 (min)
S. oralis	1.44 ± 0.0006	60
S. aureus	0.74 ± 0.02	31
E. coli	0.45 ± 0.008	19
E. faecalis	0.91 ± 0.008	38

Figure 2: *Correlation lines.* BioTimer assay enumerates bacteria in planktonic and biofilm form through the detection of the time required for an indicator switch induced by a metabolic product specific for each bacterial genus. The time required for the indicator switch is correlated to the initial number of bacteria (N) through a genus-specific correlation line described by the following equation: $t^* = -a \log N + b$, where a is a function of the metabolic product responsible for the reagent switchingm and is inversely related to bacterial growth rate k by the formula: $a = -1/k$.



Reagent with resazurin:
initial color (left, blue) and after switching (right, pink).



Spectrophotometric detection:
initial and switched reagent (solid and dotted line, respectively)

Figure 3: *BioTimer reagent to count non-fermenting bacteria.* BTA utilizes an original, appropriate reagent containing a colorimetric indicator able to switch as a consequence of the metabolism of non-fermenting bacteria in planktonic and biofilm lifestyle

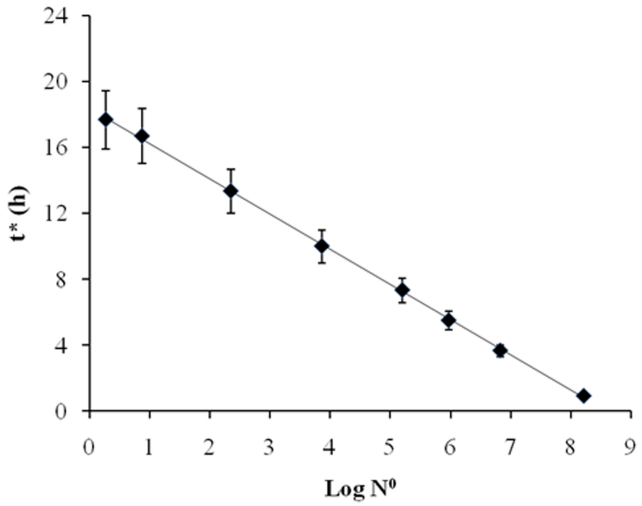
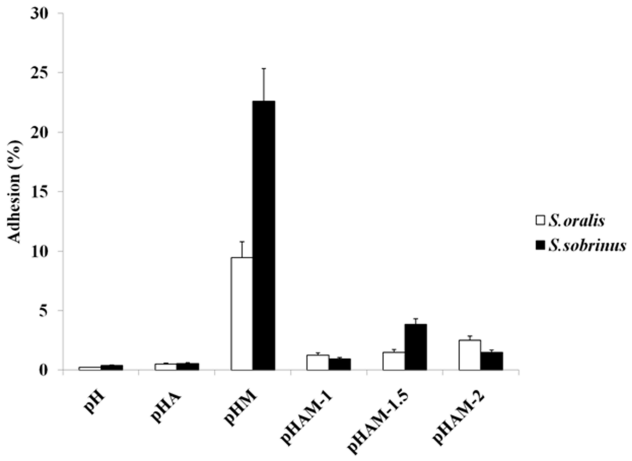


Figure 4: *Correlation line of non-fermenting bacteria.* The time required for indicator switch induced by *Pseudomonas aeruginosa* metabolism is correlated to the initial number of bacteria (N) through the specific correlation line $t* = -alogN * b$ where $a = -0.4675$ and $b = 8.5841 (R^2 = 0.9996)$.



Legend:Polymers: pH: p(HEMA); pHA: p(HEMA-co-AMPS) (10:1); pHM: p(HEMAco-METAC) (10:1); pHAM-1: p(HEMA-co-AMPS-co-METAC)(10:1:1); pHAM-1.5: p(HEMA-co-AMPS-co-METAC) (10:1:1.5); pHAM-2: p(HEMA-co-AMPS-co-METAC) (10:1:2).

Figure 5: *Adhesion efficiency of Streptococcus oralis and Streptococcus sobrinus to different dental polymers.*

	CFU counts		BIOTIMER	
FOOD SAMPLES	Mean value CFUs/g	Time (hours)	Mean value CFUs/g	Time (hours)
Ground meat	760	48	1300	14
Cheese	14	48	20	15
Fresh cow's milk cheese	1500	48	2500	12.5
Spices	4600	72	7000	8.0
Chicken	140	72	175	14
Vegetables	1300	48	1400	13

Figure 6: *Enumeration of bacterial biofilm in foods.*Comparative analysis between CFU counts and BioTimer assay in *Escherichia coli* quantitative detection.

BTA METHOD APPLIED IN ANTIBIOTIC SUSCEPTIBILITY TESTING		PLANCTONIK	BIOFILM		
		24 H	24 H	48 H	72 H
		MIC (µg/ml)	MBIC (µg/ml)	MBIC (µg/ml)	MBIC (µg/ml)
S. aureus 537	Ampicillin	0.5	2	2	2
	Gentamicin	0.5	8	8	8
	Azithromycin	8	16	16	8
	Ofloxacin	2	8	8	8
S. aureus ATCC 6538	Ampicillin	0.5	2	2	2
	Gentamicin	0.5	8	8	8
	Azithromycin	8	16	16	8
	Ofloxacin	2	8	8	4
S. aureus SA1	Ampicillin	1	2	2	4
	Gentamicin	1	4	8	8
	Azithromycin	4	16	16	16
	Ofloxacin	0.5	0.5	0.5	2
S. epidermidis 1A	Ampicillin	2	16	32	32
	Gentamicin	1	8	8	8
	Azithromycin	8	32	32	32
	Ofloxacin	8	250	250	250
S. epidermidis SEP1	Ampicillin	16	62	62	62
	Gentamicin	125	250	500	500
	Azithromycin	16	32	32	32
	Ofloxacin	250	250	1000	>1000
S. epidermidis SEP2	Ampicillin	16	62	62	62
	Gentamicin	>1000	>1000	>1000	>1000
	Azithromycin	8	32	32	32
	Ofloxacin	250	250	1000	>1000

Figure 7: *BioTimer assay in detecting antibiotic susceptibility of Staphylococcus spp adherent in biofilm.*

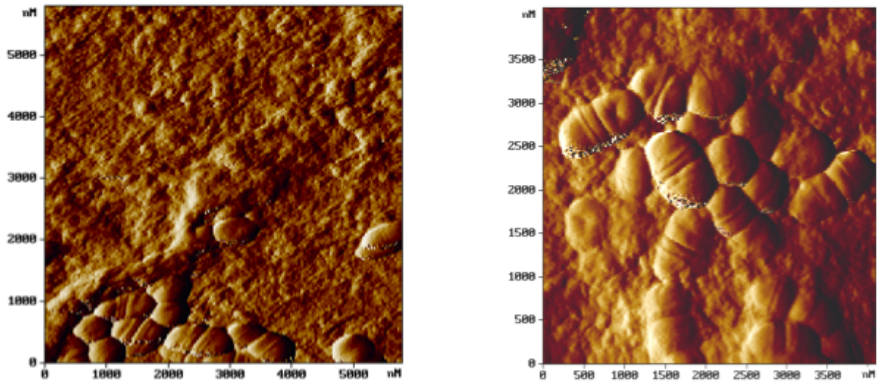


Figure 8: *AFM images of nano-structured glass bead colonized by Streptococcus mutans biofilm.*