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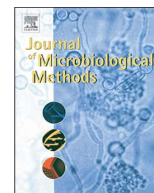

Biotimer assay: A reliable and rapid method for the evaluation of central venous catheter microbial colonization

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ABSTRACT

Adherent bacteria and biofilm frequently colonize central venous catheters (CVCs). CVC colonization is correlated to infections and particularly to bloodstream ones. The classical microbiological methods to determine of CVC colonization are not fully reliable and are time-consuming. BioTimer Assay (BTA) is a biological method already used to count bacteria adherent to abiotic surfaces and biofilm without sample manipulation. BTA employs specific reagents whose color changed according to bacterial metabolism. BTA is based on the principle that a metabolic reaction will be faster when more bacteria are present in the sample. Therefore, the time required for color changes of BTA reagents determines the number of bacteria present in the sample through a correlation line. Here, for the first time, we applied BTA and a specifically developed laboratory procedure to evaluate CVC colonization in comparison with the routine microbiological method (RMM). 125 CVCs removed from patients for suspected catheter-related bloodstream infection (CRBSI) or at hospital discharge were examined. BTA was reliable in assessing sterility and CVC colonization (100% agreement with RMM) and in recognizing the presence of fermenting or non-fermenting bacteria (97.1% agreement with RMM) shortening the analytical time by between 2- and 3-fold. Moreover, the reliability of BTA as early alert of CRBSI was evaluated. The sensitivity, specificity, positive, and negative predictive values for BTA as an early alert of CRBSI were 100, 40.0, 88.8 and 100%, respectively.

In conclusion, BTA and the related laboratory procedure should be incorporated into routine microbiological methods since it can be considered a reliable tool to evaluate CVC colonization in a very short time and a rapid alert for CRBSIs.

1. Introduction

Central venous catheters (CVCs) are a mainstay for management of critically ill patients. However, CVCs may be colonized on intra- and extra-luminal surfaces by microorganisms in adherent and biofilm lifestyle (Dobbins et al., 2003; Gominet et al., 2017). CVC colonization is correlated to infectious episodes and, in particular, the catheter-related bloodstream infections (CRBSIs) are characterized by high rates of morbidity and mortality (Pratt et al., 2001; Chopra et al., 2013; Gahlot et al., 2014; Yousif et al., 2015). Moreover, the biofilms colonizing CVCs show antimicrobial resistance significantly higher than the planktonic counterparts (Donlan and Costerton, 2002; Hall-Stoodley et al., 2004; Pantanella et al., 2008) prolonging the length of hospitalization and increasing healthcare costs (Kaye et al., 2014; Gahlot et al., 2014).

The microbiological methods for the routine analysis of CVC colonization are semi-quantitative and actually based on the roll-plate technique (Maki et al., 1977; Mermel et al., 2009) or on the detachment of adherent microbes by vortex or sonication and the count of the detached microorganisms by the colony forming unit (CFU) technique (Cleri et al., 1980; Sherertz et al., 1990; Wengrovitz et al., 1991). Unfortunately, no one of these methods is fully reliable. Indeed, the roll-plate technique does not determine the intra-luminal colonization of CVCs (Dobbins et al., 2003; Mermel et al., 2009; Guembe et al., 2016; Gominet et al., 2017) and the vortex or sonication methods do not ensure the detachment of all microbes (Pantanella et al., 2013; Freitas

Abbreviations: BTA, BioTimer Assay; CVCs, central venous catheters; RMM, routine microbiological method; CRBSI, catheter-related bloodstream infection

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[1] This manuscript is dedicated to Maria Cristina Ghezzi who prematurely passed away.

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et al., 2014). It is evident that a rapid and reliable microbiological method for the early determination of CVC colonization is extremely important. BioTimer Assay (BTA) is a biological method that counts adherent bacteria and biofilm without sample manipulation as vortex or sonication to detach biomass and cultivation, thus overcoming the above-mentioned limits (Berlutti et al., 2003; Pantanella et al., 2008; Pantanella et al., 2011; Berlutti et al., 2014; Srivastava and Bhargava, 2016). In particular, BTA is based on the principle that a metabolic reaction will be faster when more bacteria are present in the sample. BTA employs specific reagents whose color changes according to bacterial metabolism. The time required for color changes of BTA reagents is inversely related to initial bacterial concentration. Therefore, the time for color change determines the number of bacteria present in the sample at Time 0 through genus-specific correlation lines. In addition, BTA was successfully employed to evaluate biofilm colonization of medical devices (Hess et al., 2011; Wells et al., 2011; Romeo et al., 2015).

Here, for the first time, BTA was applied to evaluate microbial colonization of CVCs removed from patients hospitalized at the University Hospital Policlinico Umberto I, Rome, Italy. For this purpose, a specific laboratory procedure was developed. The performance of BTA and the related laboratory procedure was compared with the method of Cleri et al. (1980) routinely employed at the Clinical Microbiological Laboratory of the same hospital. Furthermore, the correlation between BTA results and CVC-related bloodstream infection (CRBSI) diagnosis was investigated. The data presented strongly indicate that BTA can be usefully employed to evaluate CVC colonization and can represent an alert tool for CRBSI.

2. Materials and methods

2.1. Bacterial strains and culture media

Staphylococcus epidermidis ATCC 12228 and *Pseudomonas aeruginosa* ATCC 15692 (PAO1) were streaked on 5% Columbia blood agar (CBA) plates (Oxoid LTD, England) before the experiments to check purity.

2.2. BioTimer Assay (BTA)

BioTimer Assay (BTA) allows counting bacteria in planktonic, adherent and biofilm lifestyle (Berlutti et al., 2003; Pantanella et al., 2008; Pantanella et al., 2011; De Giusti et al., 2011; Berlutti et al., 2014). BTA employs two specific reagents: BioTimer-phenol red (BT-PR) and BioTimer-resazurin (BT-RZ) whose color changes due to microbial metabolism (Berlutti et al., 2003; Pantanella et al., 2008; Pantanella et al., 2011). In particular, BT-PR reagent changed red-to-yellow due to reagent acidification when inoculated with fermenting bacteria and BT-RZ reagent changed blue-to-pink due to redox reaction when inoculated with fermenting or non-fermenting bacteria (Pantanella et al., 2011). BT-PR and BT-RZ reagents were prepared as previously described with slight modification (Pantanella et al., 2008; Pantanella et al., 2011; Berlutti et al., 2014). Briefly, to prepare BR-PR reagent, 3.7 g of Brain Heart Infusion (BHI; Oxoid Ltd., UK) were dissolved in 940 ml of distilled water. After sterilization at 115 °C for 15 min, 50 ml of 10% filtered glucose solution and 10 ml of filtered 0.25% phenol red (Sigma Aldrich, Italy) were added. If necessary, the pH was adjusted to 7.2 ± 0.1. The final BT-PR reagent appeared clear and red. To prepare the BT-RZ reagent, 10 ml of freshly prepared and filtered 0.1% aqueous resazurin solution (Sigma-Aldrich, Italy) was added to 990 ml sterile BHI broth prepared as above described (Oxoid). If necessary, the pH was adjusted to pH 7.0 ± 0.1. The final BT-RZ reagent appeared clear and blue.

The time required for color change of BTA reagents is correlated to initial bacterial concentration by correlation lines. To draw the correlation lines, serial two-fold dilutions of planktonic overnight broth cultures of *S. epidermidis* and of *P. aeruginosa* in 1 ml of BT-PR and BT-RZ reagents, respectively, were performed in 24-well plates (BD, Italy), and simultaneously counted using the colony forming unit (CFU) method. Briefly, to carry out the CFU method, bacterial suspensions were diluted in sterile saline solution and plated on BHI plates. After 24 h of incubation, colonies were counted. The number of CFUs in the initial bacterial suspensions was determined by multiplying the number of counted CFUs on BHI plates with the dilution factor. The time (in hours) required for color changing of the inoculated BT-PR and BT-RZ reagents was recorded and plotted versus the corresponding CFU values (Fig. 1). The equations and the linear correlation coefficients describing the correlation lines were calculated for each microorganism on the whole data set and were: $y = -0.5045x + 7.6959$ $R^2 = 0.9891$ for *S. epidermidis* and $y = -0.4675x + 8.5421$ and $r^2 = 0.9968$ for *P. aeruginosa*. As the correlation lines correlated the time for the color change of BTA reagents with the number of planktonic CFUs, the number of bacteria in biofilm was expressed as planktonic-equivalent CFUs (PE-CFUs) (Pantanella et al., 2008).

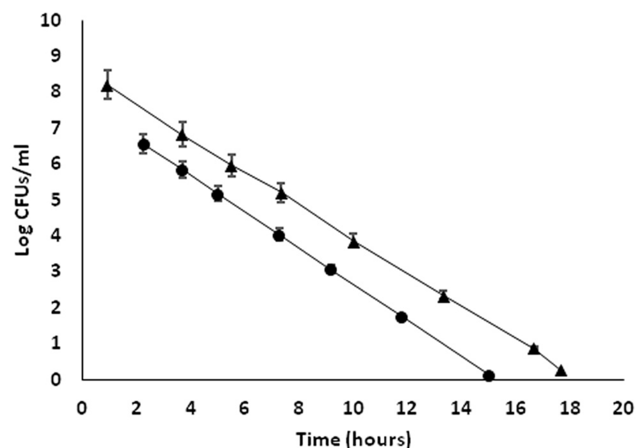

Fig. 1. BioTimer Assay correlation lines.
Legend: Correlation lines of *S. epidermidis* ATCC 12228 (circles) and of *P. aeruginosa* ATCC 15692 (triangles) in BT-PR and BT-RZ reagents, respectively.

2.3. Experimental colonization of central venous catheters

Sterile CVCs (Multi-Lumen Central Venous Catheterization Set, Arrow Italy) were aseptically cut in 1 cm-segments and immersed in sterile saline (0.9% NaCl) supplemented with 2% human serum pooled from healthy volunteers. After 30 min incubation, the CVC pieces were washed three times with sterile saline and transferred into sterile 24-well flat-bottom plates. A total of 1 ml of BHI broth containing about 10^4 CFUs of planktonic log phase cultures of *S. epidermidis* ATCC 12228 or *P. aeruginosa* ATCC 15692 (PAO1) were added to each well. CVCs were incubated for 2 and 24 h to have adherent bacteria and biofilm, respectively. After incubation, bacteria were counted using the culture method according to Cleri et al. (1980) with slight modification. Briefly, the CVC segments were transferred into sterile tubes containing 1 ml of BHI broth and then vortexed for 30 s to detach bacteria. Detached bacteria were counted by CFU method on BHI agar. To evaluate the residual adherent bacteria after vortex, the CVC segments were immersed in 1 ml of BT-RP and BT-RZ reagents and counted by BTA.

2.4. Central venous catheters collection

CVCs were randomly selected among those received at the Clinical Microbiology laboratory at “Azienda Policlinico Umberto I”, Hospital of Sapienza University of Rome, Italy during a two years period (March 2014 to March 2016). CVCs were removed from hospitalized patients either at the end of the clinical therapy or for the clinical suspicion of CRBSI (Mermel et al., 2009).

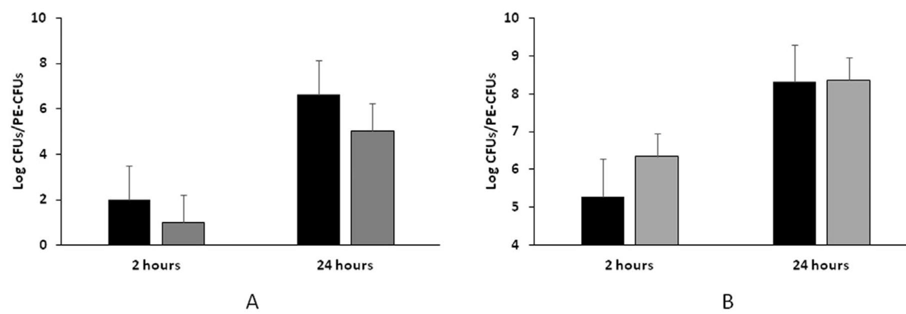

Fig. 2. Counts of *Staphylococcus epidermidis* ATCC 12228 and *Pseudomonas aeruginosa* ATCC 15692 adherent on experimentally colonized central venous catheters.
Legend: colonized central venous catheters were vortexed for 30 s and detached bacteria were counted and expressed as CFUs (black columns); the residual adherent bacteria after vortex were counted using BioTimer Assay and expressed as planktonic-equivalent CFUs (PE-CFUs) (grey columns) (for details see M & M section); panel A: *S. epidermidis*; panel B: *P. aeruginosa*.

2.5. Laboratory procedures

2.5.1. Routine microbiological method (RMM)

CVCs were examined at the Clinical Microbiology Laboratory by RMM according to Cleri et al. (1980) with slight modification. In brief, approximately 5 cm of the tip distal part of the CVCs was placed in 1 ml of BHI broth and vigorously shaken on a vortex for 30 s. A volume of 10 μl of the suspension was then streaked on 5% Columbia Blood Agar (CBA) plates (Oxoid Ltd., UK). After incubation at 37 °C for 24 h, the number of CFUs was determined. The CBA negative plates were incubated for further 24 h to assess the sterility of the culture. The identification of the isolates was performed using the Vitek 2® automated method (Biomerieux Diagnostic System, Inc., St. Louis, MI). CVC colonization was defined when $\geq 10^2$ CFUs/ml of S. *epidermidis* or coagulase-negative *Staphylococcus* (CoNS) were counted or when microbes other than S. *epidermidis* or CoNS were isolated irrespective of the bacterial count.

2.5.2. BTA laboratory procedure

After the vortex treatment (see above), the 5 cm-tip distal part of the CVCs were sent to Microbiology Laboratory at Department of Public Health and Infectious Diseases, Sapienza University of Rome, Italy to be examined by BTA and related procedure. The CVCs were cut in two identical pieces of 2.5 cm and completely immersed in 5 ml of BT-PR and BT-RZ reagents. The BTA reagents were incubated at 37 °C without agitation and the color was monitored every 30 min during the first 8 h and then every hour for a total of 24 h. The time required for color changing was recorded and used to evaluate the number of PE-CFUs. In particular, the changing of BT-PR alone or of both BT-PR and BT-RZ reagents indicated the presence of fermenting microbe(s) in pure or mixed flora. In this case, the *S. epidermidis* correlation line was used to calculate the PE-CFUs. The changing of the sole BT-RZ reagent indicated the presence of non-fermenting microbe(s) and hence the correlation line of *P. aeruginosa* was used to evaluate the PE-CFUs. The BT-PR and BT-RZ inoculated reagents, whose color did not change within the first 24 h of incubation, were considered sterile.

2.6. Statistical analysis

Mean values and standard deviations were calculated on whole data sets obtained from at least three independent experiments. Correlation lines were obtained by linear regression analysis, and linear correlation coefficients were calculated from the equation: $r = (n\Sigma xy - \Sigma x\Sigma y) / (\text{sqrt}((n\Sigma x2 - (\Sigma x)2)(n\Sigma y2 - (\Sigma y)2)))$. The sensitivity, specificity, positive predictive (PPV), and negative predictive values (NPV) for BTA as a rapid alert of CRBSI were evaluated.

2.7. Ethics

Written patient consents were not required because of the observational nature of this study. The CVCs were removed by the healthcare professionals at the hospitalization site of the patients and sent to the laboratory for analysis, and therefore the authors did not collect clinical samples. Patient data concerning microbiological analysis of CVC and blood samples were collected from an electronic database. Laboratory staff members not involved in the study anonymized these records.

3. Results

3.1. Experimental colonization of CVCs

Experimental colonization of CVCs was performed to validate BTA in counting adherent bacteria to CVCs. CVC colonization was evaluated counting both the vortex-detached bacteria by CFU method and the residual adherent bacteria after vortexing by BTA. Other achieved aims were to determine if the vortex procedure did not completely detach *S. epidermidis* and *P. aeruginosa* (Fig. 2, black columns) and that BTA was able to count the residual adherent *S. epidermidis* and *P. aeruginosa* (Fig. 2, grey columns).

Therefore, BTA was judged reliable and it was employed in analyzing CVCs removed from the hospitalized patients.

3.2. Evaluation of bacterial colonization of CVCs

During the two-year period, 125 CVCs were analyzed of which 29 were removed for suspicion of CRBSI and 96 at the hospital discharge. Out the CVCs, 90 were considered sterile using RMM since no microbial growth was observed on CBA plates after 48 h of incubation. These CVCs were considered sterile also for BTA, as they not induced the color change of BTA reagents within 24 h of incubation (100% agreement with RMM).

Out of the 35 non-sterile CVCs, 29 were all those removed for suspicion of CRBSI and six were removed at hospital discharge (Table 1).

According to RMM (Table 1), 27 CVCs gave rise to the development of microbial colonies after 24 h of incubation, and eight after 48 h. In 30 cases, the counts were higher than 10^2 CFUs/ml and the CVCs were considered colonized. In five cases counts lower than 10^2 CFUs/ml were determined. Of those CVCs, two were considered colonized since *Alcaligenes* spp., *Serratia* spp. and *Corynebacterium* spp. were isolated and three were considered as non-colonized since CoNS or *S. epidermidis* were identified. Totally, RMM recognized as colonized 32 CVCs.

Using BTA (Table 1), all 35 CVCs determined the color change of one or both BTA reagents within 24 h of incubation indicating the presence of adherent microbes (100% agreement with RMM). The bacterial counts ranged from $7.6 * 10^6$ to $\leq 10^2$ PE-CFUs. In particular, 30 CVCs showed counts higher and five lower than 10^2 PE-CFUs. Applying the criteria of the RMM, BTA correctly recognized as colonized 32 CVCs (100% agreement).

In addition to bacterial count, BTA method allowed evaluating the presence of fermenting and non-fermenting microbes through the observation of the color change of BTA reagents (Table 1). Non-fermenting bacteria changed BT-RZ reagent and fermenting microorganisms in pure or in mixed flora with non-fermenting bacteria changed both BT-PR and BT-RZ reagents. In particular, seven CVCs changed BT-RZ reagent only indicating the presence of non-fermenting bacteria,

Table 1
Performance of BioTimer Assay in comparison to the routine microbiological method in evaluating CVC colonization.

CVC[a]		BioTimer Assay			Routine microbiological method	
Acronym	Removed for	Changing time[b] (hours)	PE-CFUs/CVC[c]	F/NF[d]	Colonization[e]	Microbe identification[f]
DE	S-CRBSI	3	$7.6 * 10^6$	F	Y	*Pseudomonas* spp., *Enterococcus faecalis*, *Proteus mirabilis*, CoNS
CG	S-CRBSI	3.5	$3.2 * 10^7$	NF	Y	*Acinetobacter baumanni*, *Serratia marcescens*
DAE	S-CRBSI	4	$2.4 * 10^6$	F	Y	*Staphylococcus aureus*, *A. baumanni*, *P. vulgaris*
LR	S-CRBSI	5	$7.4 * 10^5$	F	Y	*Klebsiella pneumoniae*
MM	S-CRBSI	5	$7.4 * 10^5$	F	Y	*E. faecium*
FD	S-CRBSI	5.5	$4.2 * 10^5$	F	Y	*Klebsiella* spp., *Candida* spp.
CA	S-CRBSI	6	$2.3 * 10^5$	F	Y	*Enterococcus* spp., *Klebsiella* spp., *Pseudomonas* spp.
PM	S-CRBSI	6	$2.2 * 10^6$	NF	Y	*P.aeruginosa*
ML	S-CRBSI	6.5	$1.3 * 10^5$	F	Y	*A. baumanni*, *Pseudomonas* spp., *Escherichia coli*
ZA	S-CRBSI	6.5	$1.3 * 10^5$	F	Y	*S. aureus*
CP	S-CRBSI	7	$7.5 * 10^5$	NF	Y	*A. baumanni*, *Pseudomonas* spp.
MAL	S-CRBSI	7	$7.5 * 10^5$	NF	Y	*A. baumanni*, *Pseudomonas* spp.
MA	S-CRBSI	7	$7.3 * 10^4$	F	Y	*S. aureus*
BV	S-CRBSI	8	$2.5 * 10^5$	NF	Y	*A. baumanni*
CR	S-CRBSI	8	$2.3 * 10^4$	F	Y	CoNS
PAM	S-CRBSI	8	$2.3 * 10^4$	F	Y	*Candida* spp.
DO	S-CRBSI	8	$2.3 * 10^4$	F	Y	*C. albicans*
MIR	S-CRBSI	8	$2.3 * 10^4$	F	Y	*E. coli*
CM	S-CRBSI	11	$7.0 * 10^2$	F	Y	CoNS
LD	S-CRBSI	11	$7.0 * 10^2$	F	Y	CoNS
AG	S-CRBSI	11	$7.0 * 10^2$	F	Y	CoNS
RA	S-CRBSI	11	$7.0 * 10^2$	F	Y	*S. epidermidis*
CAG	HD	11	$7.0 * 10^2$	F	Y	*S. epidermidis*
BA	S-CRBSI	12	$2.2 * 10^2$	F	Y	CoNS
AD	S-CRBSI	12	$2.2 * 10^2$	F	Y	*K. ornitholytica*, CoNS
GV	S-CRBSI	12	$2.2 * 10^2$	F	Y	*C. albicans*
CEC	S-CRBSI	12	$2.2 * 10^2$	F	Y	*CoNS*
MOR	S-CRBSI	12	$3.4 * 10^3$	NF	Y	*A. baumanni*
PA	HD	12	$2.2 * 10^2$	F	Y	*Candida* spp.
PRO	S-CRBSI	13	$1.2 * 10^3$	NF	Y	*A. baumanni*, *Pseudomonas* spp.
RM2	HD	13	$< 10^2$	F	N	CoNS
DIE	HD	15	$< 10^2$	F	N	*S. epidermidis*
DE2	HD	15	$< 10^2$	F	N	CoNS
RM	S-CRBSI	18	$< 10^2$	F	Y	*Alcaligenes spp.*, *Serratia spp.*
GON	HD	18	$< 10^2$	F	Y	*Corynebacterium* spp.

[a] CVC: central venous catheters were removed for suspected catheter-related bloodstream infection (S-CRBSI) or at hospital discharge (HD).
[b] Changing time of BioTimer Assay reagent(s).
[c] Microbial counts were calculated using *S. epidermidis* and *P. aeruginosa* correlation lines and expressed as PE-CFUs (see 2.2 section for details).
[d] F: changing of BT-PR reagent indicating the presence of fermenting microbe(s); NF: changing of BT-RZ reagent only indicating the presence of non-fermenting microbe(s).
[e] Y, colonized CVC; N: non-colonized CVC.
[f] *Staphylococcus* identification was done at level species for *S. aureus* and *S. epidermidis* only. Staphylococci other than *S. aureus* and *S. epidermidis* are indicated as coagulase-negative staphylococci (CoNS).

while 28 CVCs determined the change of both BTA reagents indicating the presence of fermenting microbes (Table 1). Interestingly, *Candida* spp. also changed BTA reagent like to fermenting bacteria do. The presumptive identification of fermenting or non-fermenting micro-organisms was confirmed in 34 out 35 cases (Table 1) (97.1% agreement with RMM). One discrepancy was observed for CG CVC. In this case, BTA indicated the presence of non-fermenting bacteria while it was actually colonized by non-fermenting *Acinetobacter* spp. and fermenting *Serratia* spp.

3.3. Time required for the evaluation of sterility and colonization of CVCs

The time necessary for the evaluation of sterility and colonization of CVCs using the RMM and BTA was compared (Table 2).

The time required to analyze all CVCs was shorter using BTA than RMM (63% of reduction). In particular, the time needed for the determination of sterility was reduced by 50% and that for the evaluation of CVC colonization to about 67%.

3.4. BTA as early alert of CRBSI

Finally, we evaluated the reliability of BTA as an early alert of CRBSI. Therefore, the microbiological data of the 29 CVCs removed for suspicious of CRBSI as well as the companion blood cultures carried out to confirm the CRBSI diagnosis according to the criteria of Mermel et al. (2009) were retrospectively analyzed. Out of the 29 CVCs, 24 were actually associated with CRBSI and five were not (Table 3).

The microbial counts of the CVCs removed for suspicion of CRBSI as determined by BTA ranged from $7.6 * 10^6$ to $< 10^2$ PE-CFUs and induced the change of BTA reagents in 3–18 h (Table 3). In particular, 18 CVCs induced the color change of BTA reagents within 8 h (microbial load ranging from $3.2 * 10^7$ to $2.3 * 10^4$ PE-CFUs) and all were

Table 2
Time required for the evaluation of central venous catheter sterility and colonization using BioTimer Assay and routine microbiological method.

CVCs[a]	No.	Hours	
		BioTimer Assay	Routine microbiological method
Sterile	90	2160	4320
Nonsterile	35	309	1032
Total	125	2469	5352

[a] CVCs: central venous catheters.

Table 3
Correlation between the performance of BioTimer Assay in counting microorganisms colonizing central venous catheters and catheter-related bloodstream infection.

CVCs[a] (no.)	BioTimer Assay		CRBSI[d]	
	Changing time[b] (hours)	PE-CFUs/CVC[c] (range)	Confirmed	Non confirmed
3	≤ 4	$3.2 * 10^7$–$2.4 * 10^6$	3	0
5	5–6	$2.2 * 10^6$–$1.3 * 10^5$	5	0
10	7–8	$7.5 * 10^5$–$2.3 * 10^4$	10	0
9	11–12	$3.4 * 10^3$–$2.2 * 10^2$	6	3
2	≥ 12	$1.2 * 10^3$– $< 10^2$	0	2
Total 29			24	5

[a] CVCs: central venous catheter.
[b] Changing time of BioTimer Assay reagent(s).
[c] Microbial counts were calculated using *S. epidermidis* and *P. aeruginosa* correlation lines and expressed as PE-CFUs (See Section 2.2 for details).
[d] CRBSI: catheter-related bloodstream infection.

associated with CRBSI. Among the nine CVCs inducing the change of BTA reagents between 11 and 12 h (microbial load ranging from $3.4 * 10^3$ to $2.2 * 10^2$ PE-CFUs), six were associated with CRBSIs (66.6% of CVCs) and three were not (33.3% of CVCs). Finally, the two CVCs that induced the change of BTA reagent in $>$ 12 h (microbial load of $1.3 * 10^3$ and $< 10^2$ PE-CFUs) were not associated with CRBSI. Based on these observations, we evaluated the possibility of considering BTA as a rapid alert of CRBSIs. In this respect, the 12 h-time was considered as the cut-off to discriminate between the possible and non-possible association of the changing time of BTA reagents and CRBSI. Therefore, we calculated that with respect to the diagnosis of CRBSI, BTA showed the sensitivity equal to 100%, specificity of 40.0%, positive and negative predictive values equal to 88.8 and 100%, respectively. Taking together these data, we had strong evidence that this novel biological method can actually represent an early alert of CRBSI.

4. Discussion

Previously, BTA was proven reliable to count microbial biofilm adherent to abiotic surfaces (Pantanella et al., 2008; De Giusti et al., 2011; Berlutti et al., 2014). Of note, BTA is performed without sample manipulation thus overcoming the bias related to CVC intra-luminal colonization and to vortex or sonication procedures (Freitas et al., 2014; Pantanella et al., 2013; Srivastava and Bhargava, 2016). In particular, vortex procedures do not guarantee the detachment of all microbes as confirmed by the data reported here (Fig. 2). As matter of fact, BTA revealed residual adherent *S. epidermidis* and *P. aeruginosa* after vortexing. Of note, the counts of residual adherent bacteria were similar or higher than those of detached ones. This observation may be of interest since the microbial count is a critical parameter in recognizing CVC colonization.

BTA was applied to analyze the CVCs removed from hospitalized patients in comparison to RMM. In particular, BTA allowed: i) the determination of the sterility of CVCs; ii) to count bacteria adherent to CVCs; iii) to assess the presence of fermenting or non-fermenting microbes; iv) to have an early response.

Firstly, BTA correctly recognized as sterile or non-sterile all CVCs since no false positive and negative result was recorded (100% concordance with RMM). The count of the adherent microbial population is of paramount importance, as this quantitative criterion coupled with microbial identification allowed the recognition of the colonized CVCs (Mermel et al., 2009). It should be underlined that BTA was performed after RMM that detached microorganisms from CVCs. If only BTA is performed, it seems reasonable to expect higher microbial counts than that obtained. In any case, applying the criteria of RMM (i.e.: microbial count and identification), BTA correctly recognized all colonized CVCs (100% agreement with RMM).

As noted above, BTA differentiated between fermenting and non-fermenting bacteria (Table 1). In this respect, BTA gave correct information in 34 out 35 CVCs (97.1% agreement). The sole discrepancy concerned CG CVC that was colonized by both non-fermenting and fermenting bacteria as determined by RMM while BTA analysis indicated the presence of non-fermenting bacteria. This discrepancy may be due to the different proportion of non-fermenting and fermenting bacteria being the first the most abundant. It should be recalled that *Candida* spp. changed BTA reagents similarly to fermenting bacteria. Even if the BTA reagents were not devoted to counting *Candida* spp., this result strongly suggests that BTA-PR reagent can be applied to count *Candida* spp. thus hypothesizing a further application of BTA.

An additional relevant performance of BTA was the promptness of response. Indeed, the time needed to recognize sterile CVCs was shortened to about 2.0-fold in comparison to RMM (24 vs 48 h). To evaluate if the 24 h-time of incubation was actually reliable to assess CVC sterility, the non-changed BTA reagents were further incubated. The color of BTA reagents did not change after 48 h of incubation thus confirming that 24 h of incubation was reliable in determining CVC sterility. Therefore, the 24 h-time could be considered also the cut-off time to discriminate between sterile and non-sterile CVCs. As a consequence, using BTA the bacterial counts of non-sterile CVCs were obtained within 24 h thus shortening the time to recognize non-sterile CVCs to about 3-fold (24 vs 72 h).

Finally, a peculiar characteristic of BTA performance was related to the correlation between the changing time of BTA reagents and CRBSI. Bloodstream infections must be diagnosed in a timely manner so that adequate antimicrobial therapy can be started (Watson and Al-Hasan, 2014; Buehler et al., 2016). Unfortunately, the blood culture is time-consuming due to the necessity of microbial growth while BTA does not require bacterial growth since it is based on the measure of microbial metabolism. Interestingly, 100% and 67% of the CVCs inducing the change of BTA reagent(s) within 8 and 12 h, respectively, corresponded to CRBSI diagnosis (Table 3). Since the time of BTA response is inversely related to microbial load, a rapid change of BTA reagents indicates a high number of colonizing bacteria on CVCs thus alerting the physician to the possibility of CRBSI. Considering the cut-off of 12 h, the high values of sensitivity, positive, and negative predictive values strongly suggest a possible use for BTA as an early alert of CRBSIs.

In conclusion, even if a limited number of CVCs was examined, the data are encouraging since BTA can give reliable indications in a very short time. In our opinion, BTA and the related laboratory procedure should be incorporated into routine microbiological evaluations of CVC colonization to improve the performance of routine microbiological methods.

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