Measurement 45 (2012) 1083–1088

Contents lists available at SciVerse ScienceDirect

Measurement

journal homepage: www.elsevier.com/locate/measurement

Microtiter spectrophotometric biofilm production assay analyzed with metrological methods and uncertainty evaluation

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[c] Regione Autonoma della Sardegna grant FSE 2007-2013 L.R. 7/2007 "Promozione della Ricerca Scientifica e dell'Innovazione Tecnologica in Sardegna", Italy

ARTICLE INFO

Article history:
Received 16 September 2011
Received in revised form 9 November 2011
Accepted 31 January 2012
Available online 9 February 2012

Keywords:
Biofilm
Microtiter spectrophotometric assay
Uncertainty evaluation

ABSTRACT

The microbial biofilm is a structure often developed by microorganisms when performing their harmful effects, both in the medical and industrial fields. Therefore, methods allowing identification and analysis of the biofilm play a fundamental role in determining the kind of intervention needed to avoid these effects. The microtiter spectrophotometric assay is recognized as the gold standard method to quantify a biofilm and to analyze the anti-biofilm activity of various substances. The aim of the present work is to validate this method through an uncertainty evaluation, covering eight different microbial species.

The results show that the microtiter spectrophotometric assay is adequate to perform the biofilm analysis, with a good reproducibility and a reasonable uncertainty. However, the method requires a thorough knowledge of bio-dynamics concerning microbial species tested, in order to perform some protocol improvements catering in turn for better results.

1. Introduction

Microorganisms can live in one of two possible states: sessile or planktonic. The sessile phenotype results from attachment and usually develops into a biofilm that has unique characteristics [1]. The biofilm is commonly defined as "*an assemblage of microbial cells that is irreversibly associated (not removed by gentle rinsing) with a surface and enclosed in a matrix of primarily polysaccharide material.*" [2]. This definition is not entirely satisfactory, since a biofilm may be not only an aggregation of bacteria but also, as recently defined, "*a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription*" [3]. A biofilm is characterized by the adhesion of the cells to a non exfoliative surface, immersed in an aqueous medium and/or on other bacteria cells. The mechanism of attachment may be explained in terms of several factors, namely substratum effects, conditioning films forming on the substratum, hydrodynamics of the aqueous medium, characteristics of the medium, and various properties of the cell surface [2]. The association between biofilms and diseases is not always easy, because the biofilm infection cannot be proved according to Koch's postulates [1]. Infections strongly linked to a biofilm development, such as periodontal disease, endodontic infections, candidiasis, valve endocarditis, cystic fibrosis, urinary catheter cystitis, have all in common the resistance to non-invasive therapies (as drug therapy). The study of this microbial state is today indispensable to obtain a diagnosis and to decide an appropriate therapy [1,4–8]. Biofilm infections are often originated by nosocomial infections linked to poorly sterilized surfaces of medical devices, entailing critical consequences for involved patients [1,9,10]. Among the microbial species involved in biofilm infections are some microbes having a

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doi:10.1016/j.measurement.2012.01.033

primary role or considered model organisms for in vitro analysis, and therefore are among the most studied microbial species (spp) both in vivo and in vitro. Eight of these microbes, namely *Escherichia coli* (*Ec*), *Pseudomonas aeruginosa* (*Pa*), *Klebsiella pneumonia* (*Kp*), *Bacillus subtilis* (*Bs*), *Enterococcus faecalis* (*Ef*), *Staphylococcus aureus* (*Sa*), *Candida albicans* (*Ca*) and *Aggregatibacter actynomicetemcomitans* (*Aa*) [1,4,9–15] (Table 1), were considered in this study.

The development of a reproducible, specific and sensitive biofilm measurement method is today necessary in both medical and industrial fields. Among the various methods, indirect and direct applications may be distinguished. Indirect applications, such as standard plate counts, roll techniques, and sonication, allow the operator to obtain a quantification analysis of the biofilm after a detaching action. Other indirect techniques, such as radiolabeled bacteria, enzyme-linked immunosorbent assay, biologic assays, stained bacterial films, and microtiter plate procedures, enable the observer to obtain a quantification evaluation of the biofilm by measuring some attribute for the attached organism [16]. While many works in literature found limits often linked to the indirect methods [16–18], the direct methods show a better performance in terms of biofilm assessment, offset however by greater difficulties associated with techniques and equipment which may not be readily available (laser-scanning confocal, transmission electron and scanning electron microscopes) [16].

Table 1
Main diseases related to bacterial spp examined; nosocomial infections are identified.

Biofilm microbial species	Infection or disease	Nosocomial
Escherichia coli (*Ec*)	Biliary tract infection	NO
	Bacterial prostatitis	NO
	Orthopedic devices infection	YES
Pseudomonas aeruginosa (*Pa*)	Cystic fibrosis	NO
	Contact lens infection	YES
	Central venous catheters infection	YES
	Orthopedic devices infection	YES
Klebsiella pneumonia (*Kp*)	Urinary catheter cystitis	YES
	Central venous catheters infection	YES
Bacillus subtilis (*Bs*)	Model organism	–
Enterococcus faecalis (*Ef*)	Endodontic infection	NO
	Urinary catheter cystitis	YES
	Mechanical heart valves infection	YES
	Orthopedic devices infection	YES
	Intra-Uterin devices infection	YES
Staphylococcus aureus (*Sa*)	Arteriovenous shunts infection	YES
	Intra-Uterin devices infection	YES
	Pentile prostheses infection	YES
Candida albicans (*Ca*)	Candidiasis	NO
	Vaginitis	NO
	Peritoneal dialysis peritonitis	YES
	Vascular catheters infection	YES
	Joint prostheses infection	YES
	Central venous catheters infection	YES
Aggregatibacter actynomicetemcomitans (*Aa*)	Periodontal disease	NO
	Meningitis	NO
	Heart disease	NO

An indirect method which showed a good level of reproducibility, specificity and sensitivity, along with substantial simplicity, is the microtiter or microplate spectrophotometric assay [16,19–22]. This method, first described in 1977 [19] and modified and improved in 1998 and in 2002 [20,16], is highly adaptable to the type of organisms to be studied in various and different growing conditions, is used routinely [23,24], and is nowadays considered as the gold standard for the indirect evaluation of biofilm [22].

Spectrophotometry, a technique based on the interaction of light and matter, investigates the absorption of different substances within the wavelength range 190–780 nm. In this range the absorption of the electromagnetic radiation is caused by the excitation of the bonding and non-bonding electrons of the ions or molecules. Spectrophotometry is used for both qualitative and quantitative investigations of samples. The wavelength at the maximum of the absorption band is related to the amount of the species absorbing the light.

This work is aimed at validation of the microtiter spectrophotometric biofilm production assay as a measurement tool using a metrological approach, exploiting statistical methods in order to perform an uncertainty evaluation [25].

2. Materials and methods

2.1. Culture preparation

The following species were used for this study:

1. *Gram positive bacteria*: *Staphylococcus aureus* ATCC 6538, *Enterococcus faecalis* ATCC 29212 [26], *Bacillus subtilis* (clinical strain) [8].
2. *Gram negative bacteria*: *Escherichia coli* ATCC 7075, *Pseudomonas aeruginosa* ATCC 27853, *Aggregatibacter actinomycetemcomitans* DSM 11123 (genotype JP2) (Deutsche Sammlung von Mikrorganismen und Zellkulturen GmbH, Braunschweig, Germany) [26], *Klebsiella pneumoniae* (clinical strain) [27].
3. *Fungi*: *Candida albicans* from oral clinical isolates. These specimens were plated in Sabouraud glucose agar for 48 h at 35 °C (Microbiol, UTA, Cagliari, Italy). The colonies were identified with an API ID32C system (Biomerieux, St Louis, MO) and maintained at −20 °C in skimmed milk (Oxoid, Basingstoke, UK) [26].

Before the application of the spectrophotometric assay method, the selected microorganisms were divided into three groups: