Journal of Microbiological Methods 118 (2015) 106–112

Contents lists available at ScienceDirect

Journal of Microbiological Methods

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

Evaluation of baicalein, chitosan and usnic acid effect on *Candida parapsilosis* and *Candida krusei* biofilm using a Cellavista device

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ARTICLE INFO

Article history:
Received 8 July 2015
Received in revised form 4 September 2015
Accepted 5 September 2015
Available online 8 September 2015

Keywords:
Cellavista
Biofilm
Candida parapsilosis
Candida krusei
Baicalein
Chitosan
Inhibition
Eradication

ABSTRACT

Biofilms are often the cause of chronic human infections and contaminate industrial or medical equipment. The traditional approach has been to use increasing concentrations of antibiotics, but microorganisms rapidly develop multiresistance to them. Therefore, we investigated the use of natural substances as an alternative solution. The quantification of the biofilms based on the colonized areas was measured using a Cellavista automatic microscope equipped with image analysis software.

Using the Cellavista device brings new possibilities for qualification and quantification of sessile cells. In our study, this feature was documented by exploring the antifungal/anti-biofilm activity of amphotericin B, baicalein, chitosan and usnic acid against yeast biofilm formation.

The influence of these substances on the formation and eradication of opportunistic pathogenic yeasts *Candida parapsilosis* and *Candida krusei* biofilms was studied in 96-well polystyrene microtiter plates. While amphotericin B was not very efficient, the use of baicalein and chitosan, even in minimum inhibitory concentrations, was found to rapidly decrease the colonized areas in the wells. The usnic acid did not display any significant antibiofilm properties even at concentration 300 μg ml^{-1}. Our results propose that Cellavista is a promising tool for the study of yeast biofilm formation and the effects of antimicrobial agents.

1. Introduction

Biofilms are highly organized matrix-enclosed microbial communities that are irreversibly attached to a surface, phase interface or to each other. These microbial communities often contaminate medical instruments or industrial devices and cause a wide range of human infections. The huge problem of treating these infections is the high antimicrobial resistance of biofilms to the typically used antibiotics. Microorganisms in the biofilm are more (up to 1000-fold) resistant than planktonic cells (Thebault et al., 2013).

Almost 99% of microorganisms in nature are able to grow in the form of a biofilm, in which they colonize diverse surfaces (Thebault et al., 2013). Bacterial biofilms and their associated infections have generally been more widely explored than biofilms caused by other microorganisms. However yeast biofilms, especially *Candida* spp. biofilms, can also cause very serious infections in humans (Trofa et al., 2008, Pires et al., 2011).

Candida spp. are one of the most common isolated yeasts from nosocomial infections or from biofilms which are formed on medical instruments. *Candida albicans* is often designated as the most widespread opportunistic pathogenic yeast, but *Candida parapsilosis*, *Candida tropicalis*, *Candida glabrata* and *Candida krusei* have also become the subject of increasing attention in many studies (Kuhn et al., 2002, Gacser et al., 2007, Estivill et al., 2011). *C. parapsilosis* causes many types of diseases, particularly in immunodeficient patients. Examples of these illnesses include fungemia, endocarditis, meningitis, arthritis, ocular infections, vulvovaginitis, urinary tract infections and joint diseases (Trofa et al., 2008). While some of the *C. parapsilosis* strains are sensitive to polyene antimicrobial agents (e.g. amphotericin B, fluconazole), others are already resistant (van Asbeck et al., 2009). *C. krusei* is considered to be less pathogenic but more hydrophobic than other *Candida* spp. This factor plays an important role in the initial events leading to the colonization of host surfaces. In this context, this yeast species is able to cause a spectrum of clinical manifestations such as fungemia, endophthalmitis, arthritis and endocarditis (Samaranayake & Samaranayake, 1994).

The antibiotic still widely used against fungal infections is the polyene antibiotic amphotericin B, though it has quite serious side effects. The worst of these is nephrotoxicity, which may lead to kidney failure (Karimzadeh et al., 2013).

The attention is therefore turning to the use of natural substances with antibacterial, antifungal and antibiofilm properties that may help in the treatment of yeast biofilm infections. These substances can be used in low concentrations without negative effects on a patient and no resistance against them is developed. Examples of such natural substances include polyphenols, flavonoids, stilbenes, terpenes, polysaccharides and the active compounds of commonly used spices

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http://dx.doi.org/10.1016/j.mimet.2015.09.002
0167-7012/

(Rezanka et al., 2012). For example baicalein, a flavonoid obtained from *Scutellaria baicalensis*, has antioxidative, neuroprotective, antibacterial, antiviral and antifungal properties (Cao et al., 2008). Chitosan is a hydrophilic biopolymer, a polysaccharide which is present in the outer shell of crustaceans and has a broad antimicrobial activity (Martinez et al., 2010). Usnic acid, a secondary metabolite of lichen, exhibits antiviral, antiprotozoal, anti-proliferative, anti-inflammatory and analgesic effects (Pires et al., 2012).

A wide variety of microscopic techniques have been used to study biofilms. These methods include light microscopy, scanning laser confocal microscopy, scanning and transmission electron microscopy, atomic force microscopy, etc. (McLean et al., 2004). Optical light microscopy coupled with image analysis observes the biofilm as a two-dimensional area covered by adhered cells and is considered a fundamental method for biofilm observation. The combination of light microscopy and image analysis has been used to evaluate the numbers of cells attached, area coverage, minor applications including determination of biovolume of attached cells and observation of bacterial adhesion and biofilm growth in real time (An & Friedman, 1997).

The purpose of this paper is to report the possibility of applying the Cellavista device in a novel way as a means to study yeast biofilms and their response to antimicrobial agents. The application is demonstrated by observing the treatment of fungal biofilms with natural substances instead of antibiotics. One of the advantages of the automatic microscope Cellavista in biofilm analyses is the possibility of an automatic coupling of individual pictures and subsequent image analysis of these large surfaces. This allows a significant refinement of the final results by eliminating the natural variability of the biofilm on the one hand but also changes in the detection of different biofilm arrangements for instance due to the presence of natural substances in the environment of the biofilm on the other. Using the image analysis provided by Cellavista device, we show the positive effects of baicalein, chitosan and usnic acid in the treatment of *C. parapsilosis* and *C. krusei* biofilms and compare such treatment with the effect of amphotericin B and therefore provide evidence that Cellavista can be used as a fast, reliable tool for determining the efficiency of antimicrobial agents on biofilms.

2. Methods

2.1. Fungal strains and growth media

C. parapsilosis DBM 2165 and *C. krusei* DBM 2163 were kindly provided by the collection of microorganisms from UCT Prague. Stock cultures were stored at −70 °C and pre-cultured before each experiment aerobically at 30 °C in malt extract medium (ME). The experiments were also carried out in malt extract growth medium.

2.2. Antifungal agents

Baicalein, chitosan, usnic acid and amphotericin B were purchased from Sigma-Aldrich. Baicalein and usnic acid were dissolved in DMSO (final concentration of DMSO in medium was up to 1% in all assays) to a concentration of 0.004 and 0.03 μg ml^{-1}, chitosan was prepared by dissolving it in a small amount of acetic acid (maximally 2% of total volume) and then in sterile distilled water to a concentration of 0.073 μg l^{-1}, and amphotericin B was dissolved in sterile distilled water to a concentration of 0.029 μg l^{-1}. Each substance was stored at 4 °C until used (a maximum of one week).

2.3. Antifungal activity

Minimum inhibitory concentrations (MICs) of baicalein, chitosan, usnic acid and amphotericin B were determined by the microdilution method according to Sharma et al. (2010). The cultivation was carried out in 100-well microtiter plates using Bioscreen C analyzer (Oy Growth Curves Ab Ltd., Finland) by monitoring optical density ($OD_{wideband\ =\ 420–580\ nm}$). A volume of 30 μl of standard cell suspension of yeasts ($OD_{600\ nm} = 0.1$) was added to each well of the plates. Drug-free and yeast-free controls were included. Plates were incubated for 24 h at 30 °C. The MIC was assigned as the lowest concentration that did not allow visible growth after incubation overnight according to the definition by (Andrews, 2001). Experiments were performed with five replicate wells for each concentration.

2.4. Biofilm formation

The *C. parapsilosis* and *C. krusei* biofilms were formed on commercially available pre-sterilized, polystyrene, flat-bottomed, 96-well microtiter plates (firma). To each well of the plates, 210 μl of standard cell suspensions of yeasts in growth medium ($OD_{600\ nm} = 0.8$) and 70 μl of ME were added. Experiments were performed with sixteen replicate wells for each concentration.

2.5. Drug efficiency against adhesion

To investigate the effect of natural substances or antibiotic on initial cell adhesion one of the substances was added to the well together with standard cell suspensions of yeasts from the very beginning. The microtiter plate was covered with its lid and incubated at 30 °C, 150 rpm for 24 h.

2.6. Drug efficiency against biofilm development

To investigate the effect of natural substances or antibiotic on biofilm development, the microtiter plate with standard yeast suspensions of yeasts was cultivated for 24 h and then one of the substances was added. The wells were not washed prior to the drug addition to simulate the conditions of natural mechanisms of action in which substances interact with both adhered and suspended cells. The microtiter plate was covered with its lid and incubated at 30 °C, 150 rpm for 24 h.

2.7. Drug efficiency against pre-formed biofilms

To investigate the effect of natural substances or antibiotic on eradication of pre-formed biofilms, the microtiter plate with standard cell suspensions of yeasts was cultivated for 24 h and each well was then washed three times with saline. Then one of the substances in appropriate concentration dissolved in ME was added to each well and cultivation was carried out for an additional 24 h. The microtiter plate was covered with its lid and incubated at 30 °C, 150 rpm for 24 h.

2.8. Cellavista device

Cellavista is a fully automated cell imager that possesses brightfield and three fluorescence channels (standard set includes seven excitation wavelengths along with the respective filters). The optical system consists of four lenses: 2×, 4×, 10×, 20× and 40× and a CCD camera that provides 4 Mpixel images. The Cellavista can evaluate 6–384 microwell plates as well as Petri dishes and microscope slides.

An array of pre-defined applications is available (cell confluence, suspension cell count, cell nuclei, etc.). Each assay provides associated image processing algorithm that is defined by a particular object type that the algorithm evaluates. The parameter evaluation setting can be modified for each assay by the user.

For image obtaining the microplate is placed into the device and correct image setting can be manually adjusted (exposure, time, light intensity, gain, focus offset). The selected microplate wells are then scanned and the images are automatically coupled to produce an image of the whole well. According to the selected assay and user-defined parameters the image analysis then proceeds with automatic and consistent thresholding for each assay by the Cellavista evaluation algorithm. Depending on the assay type, different parameters can be