Journal de Mycologie Médicale xxx (2018) xxx–xxx

Available online at
ScienceDirect
www.sciencedirect.com

Elsevier Masson France
EM|consulte
www.em-consulte.com

Original article/Article original

Efficacy of modified Leeming-Notman media in a resazurin microtiter assay in the evaluation of in-vitro activity of fluconazole against *Malassezia furfur* ATCC 14521

F.E. Far [a], M.M.J. Al-Obaidi [a], M.N.M. Desa [a,b,*]

[a] *Department of Biomedical Science, Faculty of Medicine & Health Sciences, Universiti Putra, 43400 UPM Serdang, Selangor, Malaysia*
[b] *Halal Products Research Institute, Universiti Putra, 43400 UPM Serdang, Selangor, Malaysia*

ARTICLE INFO

Article history:
Received 16 January 2018
Received in revised form 11 April 2018
Accepted 12 April 2018
Available online xxx

Keywords:
Colorimetric
Fluconazole
Leeming-Notman
Malassezia furfur
Resazurin microtiter assay

ABSTRACT

Background. – *Malassezia furfur* is lipodependent yeast like fungus that causes superficial mycoses such as pityriasis versicolor and dandruff. Nevertheless, there are no standard reference methods to perform susceptibility test of *Malassezia* species yet.
Aims. – Therefore, in this study, we evaluated the optimized culture medium for growth of this lipophilic yeast using modified leeming-Notman agar and colorimetric resazurin microtiter assay to assess antimycotic activity of fluconazole against *M. furfur*.
Results. – The result showed that these assays were more adjustable for *M. furfur* with reliable and reproducible MIC end-point, by confirming antimycotic activity of fluconazole with MIC of 2 μg/ml.
Conclusion. – We conclude that this method is considered as the rapid and effective susceptibility testing of *M. furfur* with fluconazole antifungal activity.

Introduction

Malassezia spp. are the most frequent agents in both human and animals causing skin fungal infection, especially in tropical and subtropical areas [1]. *Malassezia* is a yeast that generally lives as part of human skin microflora in 75% to 98% of healthy people [2]. For the first time, Malassez (1874) described *Malassezia* as globose and elliptical budding yeasts from the stratum corneum of seborrheic dermatitis patients. Various species of *Malassezia* have been identified based on morphology, enzyme activity, growth characteristics and along with some molecular approaches [3]. It is an opportunistic polymorphic lipophilic microorganism and is distinguished from the other genus by its thick, multi-layered cell wall. Notably, *Malassezia* needs fatty acid for in-vitro growth, and its color of the colonies ranging from creamy yellow to brown white. Under specific conditions, it may vary from the saprophytic form to the pathogenic mycelia form. Also, it may cause systemic infections in immunocompromised individuals, particularly in neonates [4]. Studying the susceptibility of *Malassezia* against antimycotic drugs could provide susceptibility and resistance profile as well as potential agent for treatment [5]. Nevertheless, the standard antimicrobial testing for yeast does not fully suit the growth of *Malassezia* that requires growth supplement such as olive oil for source of fatty acids containing oleic and linoleic acid, water soluble components, vitamin and microscopic bits of olive [6]. This may complicate the minimum inhibitory concentration (MIC) determination, as the oil may not form a homogenous suspension and thus prevent fungus from being fully exposed to the antimicrobial compounds during the growing stage. Additionally, the documentation of the CLSI antifungal disk susceptibility testing method (M44-A) was available for Candida against fluconazole and voriconazole via Mueller-Hinton agar supplemented with 2% glucose and methylene blue as the test medium [3]. Unfortunately, this condition does not support the growth of lipophilic yeasts including *Malassezia* spp. [3].

Malassezia furfur is one of the important species responsible for several dermatological diseases such as dandruff/seborrhoeic dermatitis, pityriasis versicolor, and atopic dermatitis in humans [7,8]. In addition, there had also been bloodstream infections developed in patients who received parenteral lipid supplementation, caused by *M. furfur* [9]. To date, no single medium has been established to maintain all species of *Malassezia*. However, some types of media such as Leeming-Notman (LN), Dixon and PRMI

* Corresponding author. Department of Biomedical Science, Faculty of Medicine & Health Sciences, Universiti Putra, 43400 UPM Serdang, Selangor, Malaysia.
E-mail address: mnasir@upm.edu.my (M.N.M. Desa).

https://doi.org/10.1016/j.mycmed.2018.04.007
1156-5233/

1460 had been shown to support the growth of *M. furfur*t upon some modifications [10]. Previously, modified LN broth (mLNB) and modified LN agar (mLNA) were shown to exhibit some growth consistency of the species [11–13], indicating that the media may have the potential to be utilized as test medium in the antimicrobial testing. Therefore, this study was conducted to assess the suitability of common fungal media and mLNA for *M. furfur* growth and the efficacy of the mLNB in the antimicrobial susceptibility assay through a colorimetric based method (Resazurin Microtiter Assay) (REMA) using antimycotic activity of fluconazole (FLC).

Materials and methods

Microorganism

M. furfur was purchased from the American Type Culture Collection (ATCC-14521). It was cultured from frozen stock and maintained at 33 °C on Sabouraud dextrose agar (SDA) supplemented with pure olive oil. Inoculum suspension was prepared and the cell density in 0.85% saline normal was adjusted to 0.5 McFarland turbidity standard using Wickerham card method as described by Pfaller et al. [14]. Subsequently, the yeast suspension was diluted at 1:10 in a sterile saline solution (0.85%) to gain the inoculum size of 10^7 CFU. Spectrophotometric check was done at 0.18 to 0.20 at 625 nm equivalent to 3×10^8 cells/ml.

Media preparation and evaluation of growth on the different growth media

SDA, PDA (Potato dextrose agar/Difco, France) and mLNA were used with the earlier two media supplemented with olive oil (SDAO and PDAO). Two drops of pure olive oil was filtered and swabbed on the agar surface. For mLNA, the media was prepared as described by Leeming-Notman [15] and Miranda et al. [16] consisting 0.1% glucose, 0.2% yeast extract, 0.8% bile salts, 0.1% peptone, 0.1% glycerol, 3% olive oil, 0.5% Tween 60 and 50 μg/ml chloramphenicol. All the ingredients, except tween 60, lipid compounds and chloramphenicol, were mixed well in distilled water followed by pH adjustment at 6.2. The medium was then heated at near-boiling temperature. Meantime, tween 60 was heated around at 60 to 70 °C. Then, the required amounts of olive oil, glycerol and warmed tween 60 were added into the mixture and mixed moderately. After autoclaving, the molten mLNA was cooled at 45 to 50 °C followed by addition of the antimicrobial solution. All media were inoculated by swabbing with 107 CFU suspension of *M. furfur* followed by incubation for 72 hours at 33 °C. Colonial morphology of growth on agar media was observed and compared for any obvious differences.

Bioassays

Antimycotic susceptibility testing

For determination of MIC, broth micro-dilution test was carried out in conformity with the CLSI (2008) guideline in the M27-A2 document using a flat bottom 96-well plate and mLNB as the test media. The ingredient of this culture medium and inoculum suspension were prepared as previously mentioned. Final inoculum size of 1.5×10^3 was obtained after serial dilution. The FLC as a well-known antimycotic agent was used in this experiment with range of doubling concentrations tested between 0.125–64 μg/ml. After incubation at 33 °C for 48 hours, suspension that predominantly showed a decreased growth turbidity compared with negative control (drug-free growth control) was taken as the MIC. Subsequently, suspension with the concentrations of FLC next to the MIC value were plated out onto SDAO and incubated at 33 °C for 48 hours for determining the minimum fungicidal concentration (MFC).

Qualitative and quantitative colorimetric resazurin assay

The REMA was performed with slight modifications as explained by Palomino et al. [17]. In brief, the working solution of rezasurin was prepared from the resazurin sodium salt powder at 1 mg/ml concentration [18] in distilled water, and sterilized through filter before use. Following the MIC assay as described earlier, 20 μl of resazurin solution was added per well away from light (stain is sensitive to light) followed by another 24 hours incubation at 33 °C. Reading was done again at this stage for MIC which was defined as the minimum concentration of drug, which impeded change from blue (oxidized phase) to pink (reduced phase) [19]. Incubation was then further prolonged for another 24 hours. Finally, the fluorescence for all wells in the plate was determined using fluorescent microplate reader, which was set at Ex wavelength of 540 nm and Em wavelength of 590 nm. It is important to note that the background absorbance was measured in each assay to obtain the portion of absorbance that comes from the turbidity of insoluble constituents. The percentage growth or viability of *M. furfur* as inferred by the fluorescence intensity was then calculated using the equation described by Liu et al. [20].

$$\%\text{Growth} = \frac{\text{OD sample-OD corresponding background}}{\text{OD growth control-OD corresponding background}} \times 100\%$$

Statistical analysis

In the present study, all data are expressed as mean ± S.E.M. The regression analysis was performed using Statistical software program (SPSS 18) in order to find the relationship between the growth percentage of *M. furfur* and the different concentrations of FLC (μg/ml). A probability (P) value of 0.05 or less was considered as the level of significant.

Results and discussions

Comparison of three different culture media

Yeast-like fungi *M. furfur* was cultured on the different culture media; mLNA (Fig. 1a), SDAO (Fig. 1b) and PDAO (Fig. 1c) to compare the visual quality of growth with same inoculum size (10^7 CFU).

The growth properties of *M. furfur* on these three selective culture media are summarized in Table 1. The results showed that colony size of *M. furfur* on the mLNA was markedly bigger than on the SDAO and PDAO.

The SDA contains peptone as the protein source and dextrose which is a good carbohydrate source that promotes the growth of yeasts. Meanwhile, two most important compositions of PDA are potato infusion and glucose as a carbohydrate source that supports the growth of fungi and yeasts. This lipophilic yeast, *M. furfur* needs an exogenous lipid source because it is not capable of synthesizing medium-chain and long-chain fatty acids [21]. Hence, in this study, *M. furfur* was also streaked on SDA and PDA without the overlaid olive oil to determine its lipid dependency. The results revealed that *M. furfur* did not grow on SDA and PDA without the lipid overlaying onto the agar surface (data not shown), but, it grew on the SDAO and PDAO media which were covered by olive oil as a lipid source. *Malassezia* yeast was capable of consuming free fatty acids as a source of carbon; so, its growth would be instigated by

Please cite this article in press as: Far FE, et al. Efficacy of modified Leeming-Notman media in a resazurin microtiter assay in the evaluation of in-vitro activity of fluconazole against *Malassezia furfur* ATCC 14521. Journal De Mycologie Médicale (2018), https://doi.org/10.1016/j.mycmed.2018.04.007