 doi:10.20944/preprints202107.0601.v1

Communication

MalaSelect: a Selective Culture Medium for *Malassezia* Species Isolation

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Abstract: *Malassezia* species are fastidious and slow-growing yeasts whose isolation from polymicrobial samples is hampered by fast-growing microorganisms. *Malassezia* selective culture media are needed because *Malassezia* are resistant to cycloheximide, but some fungi, including the chief human commensal *Candida albicans* resist to this compound. This study aimed to test whether the macrolide rapamycin could be used in combination with cycloheximide to develop a *Malassezia*-selective culture medium. Rapamycin susceptibility testing was performed via microdilution assays in modified Dixon against *M. furfur* and five *Candida* spp. The MIC was the lowest concentration producing 90% growth inhibition. Rapamycin medium ± cycloheximide 500 mg/L was also added to FastFung solid and yeast suspensions were inoculated and incubated for 72h. Rapamycin MICs against *Candida* spp. ranged from 0.5 to 2 mg/L, except for *C. krusei* whose MIC was >32 mg/L. *M. furfur* stains were rapamycin resistant. Rapamycin and cycloheximide supplementation of the FastFung medium effectively inhibited the growth of non-*Malassezia* yeast, including the cycloheximide-resistant *C. albicans* and *C. tropicalis*. Based on our findings, we recommend using this "MalaSelect" medium for *Malassezia* isolation and culture from polymicrobial samples.

Keywords: *Malassezia*; selective culture medium; Rapamycin; isolation; polymicrobial samples.

1. Introduction

The *Malassezia* genus comprises 18 species that are lipid-dependent yeast commensals of human skin and other warm-blooded vertebrates [1]. Under certain circumstances (e.g. increased humidity and temperature), these yeast cause common human skin diseases, including pityriasis versicolor, seborrheic dermatitis, folliculitis, etc. [2]. They can also cause severe bloodstream infections in neonates or immunocompromised patients hospitalized in intensive care with parenteral nutrition [3–6]. However, data on the interactions between Malassezia species and their hosts remain scarce, especially in human. This knowledge gap has been widen because the routine use of culture media on which these lipid-dependent yeast can be cultivated is limited to a few specialized laboratory. In the literature, a variety of lipid-enriched culture media, including Dixon, Leeming-Notman agar, and their modified versions, are effective for Malassezia spp. cultivation [7–9]. Yet, these culture media are not selective in that they also allow the growth of a variety of other fungi, including Candida spp. yeast and moulds [10–13]. Relatively slow growing Malassezia spp. yeast might remain undetected due to the overgrown by faster growing, and sometimes also more abundant, fungal species present in the samples. Therefore, the use of antimycotic compounds that are ineffective against Malassezia spp. seems to be an option for the development of a selective culture medium. Among these types of compounds, Cycloheximide is widely used in culture media aimed to isolate and cultivate Malassezia spp. from clinical samples [14–17]. However, particular fungi, especially in the genus Candida (e.g. the most common human opportunistic pathogen C. albicans), are

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resistant to cycloheximide and might thus interfere with Malassezia isolation. The development of Malassezia-specific and efficient culture medium is crucial in order to be able to selectively isolate Malassezia spp. from polymicrobial clinical specimens, as these yeast species are detected at higher relatively frequencies by using culture-independent methods in complex polymicrobial niches such as the respiratory and digestive tracts [18–22].

Vézina et al. discovered rapamycin in 1975 [23]. This a secondary metabolite produced by *Streptomyces hygroscopicus* has an antifungal activity, especially against *Candida albicans*. Rapamycin is now commonly used as immunosuppressive drug [24]. Although its antifungal activity against moulds or other yeast species is well known [25,26], the potential activity of rapamycin against *Malassezia* spp. is poorly studied. However, it is recently reported that *M. furfur* and *M. sympodialis* are not sensitive to rapamycin [27]. The aim of this present study was to assess the in vitro antifungal activity of rapamycin against *Malassezia* spp. and *Candida* spp. by using broth microdilution method, and to test whether a solid culture medium supplemented with rapamycin and cycloheximide might allow growing *Malassezia* spp. while inhibiting the growth of *Candida* spp.

2. Materials and Methods

2.1. Strains

A total of 3 *Malassezia* strains, obtained from Belgian Co-ordinated Collections of Micro-organisms/Institute of Hygiene and Epidemiology (BCCM/IHEM, Sciensano, Brussels, Belgium) including *M. furfur*, *M. sympodialis* and *M. pachydermatis* were used to test the antifungal activity of rapamycin. Strains were maintained at 30°C on the FastFung medium [28,29], composed per litre [pH 6] of 43 g of Schaedler agar, 20 g of peptone, 10 g of glucose, 5 g of ox-bile, 10 g of malt extract, 2 ml oleic acid, 2.5 ml glycerol and 5 ml of Tween 60 (each from Sigma-Aldrich, Saint-Quentin Fallavier, France). Isolates of *C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. tropicalis*, and the *C. krusei* ATCC 6258 strain were also used.

2.2. Broth microdilution

Rapamycin solutions (Sigma-Aldrich, Ref. S-015-1ML) at 1 mg/ml in acetonitrile were obtained and stored at -80°C until use. The rapamycin concentration gradient tested ranged from 0.0625 to 32 mg/L. Broth microdilution method was performed by using modified Dixon broth (3.6% malt extract, 0.6% peptone, 2% ox-bile, 1% Tween 40, 0.2% glycerol and 0.2% oleic acid [pH 6]) (each from Sigma-Aldrich). Yeast inoculum suspensions of *M. furfur* and *Candida* spp. were prepared in 2 mL sterile saline solution (0.85% NaCl) and standardized spectrophotometrically at 530 nm (10^6 colony-forming units [CFU]/ml). These suspensions were diluted 1:10 in sterile distilled water and a total of 100 µL of the final dilution was transferred into a 96-well microtiter plate containing 100 µL of the medium to achieve a final concentration of 0.5–2.5 × 10^5 CFU/mL. Each assay was tested in duplicate. The microtiter plates were incubated at 30°C and visually read after 24 and 48 h of incubation. The growth of each strain at various rapamycin concentrations, as well as of a positive control cultured in rapamycin-free medium, was recorded. Sterility controls were also included in each tested strain. The MIC of each strain was defined as the lowest concentration producing 90% of inhibition when compared to the control growth.

2.1. Growth testing on Agar

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FastFung medium supplemented with 500 mg/L cycloheximide (CliniSciences, Nanterre, France) was prepared and sterilized by autoclaving at 121°C for 30 min. After cooling to approximately 56°C, FastFung medium was partitioned into two equal volumes and a solution of rapamycin was added to one volume of the FastFung medium. The two media were then distributed in sterile Petri dishes. *Malassezia* spp. and Candida spp. yeast suspensions at 106 CFU/ml were prepared and further diluted at 105, 104 and 103 in sterile distilled water. For each dilution, 20 µl was plated, as illustrated in Figure 1, onto FastFung medium with or without rapamycin. Sterile distilled water was also plated in FastFung medium as negative control. All agar plates were incubated aerobically at 30°C and examined daily for 3 days.

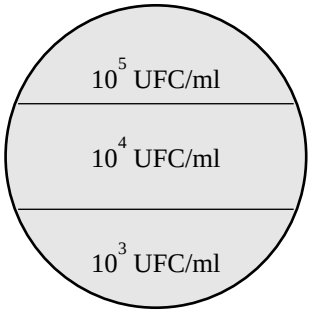

Figure 1. Plating order of different inocula for each yeast species tested.

3. Results

Rapamycin antifungal activity of was first evaluated in FastFung liquid medium (as described in Abdillah et al., submitted) against 2 M. furfur and 5 Candida strains, including *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis* and *C. krusei* ATCC 6258. The MIC results are summarized in Table 1. Rapamycin exhibited low MIC values against Candida spp. except for C. krusei ATCC 6258 whose MIC were ≥ 32 mg/l. MIC after 24h of incubation were 1 to 2-fold dilution lower than those recorded after 48 h of incubation (Table 1). Slow growing *M. furfur* required 48h reading time. Both *M. furfur* strains exhibited high rapamycin MIC ≥ 32 mg/l, suggesting that rapamycin had no significant effect against *Malassezia* spp. After determination of rapamycin MIC values against *M. furfur* and *Candida* spp., we assessed the growth of these isolates onto FastFung medium plates supplemented or not with 2 mg/L rapamycin. The plates were examined daily for 3 days. No growth was observed onto FastFung medium with cycloheximide, supplemented or not with rapamycin for *C. glabrata*, *C. parapsilosis* and *C. krusei* ATCC 6258 (Figure 2). Cycloheximide (500 mg/l) did not inhibit *C. albicans* and *C. tropicalis* in FastFung medium without rapamycin (Figure 2). In FastFung medium supplemented with both cycloheximide and rapamycin, *C. albicans* and *C. tropicalis* did not grow after 2 to 3 days of incubation. The growth of *M. furfur*, *M. sympodialis* and *M. pachydermatis* was not altered when these yeasts were cultured in FastFung medium containing cycloheximide, supplemented or not with rapamycin (Figure 2).

Table 1. Rapamycin in vitro susceptibility testing against *M. furfur* and *Candida* spp.

	Rapamycin MIC (mg/L)

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Strains	24 h	48 h
C. albicans	0.25	0.5
C. glabrata	0.5	2.0
C. tropicalis	0.25	0.5
C. parapsilosis	0.5	1.0
C. krusei ATCC 6258	32.0	> 32.0
M. furfur IHEM 17767	-	> 32.0
M. furfur IHEM 19320	-	> 32.0

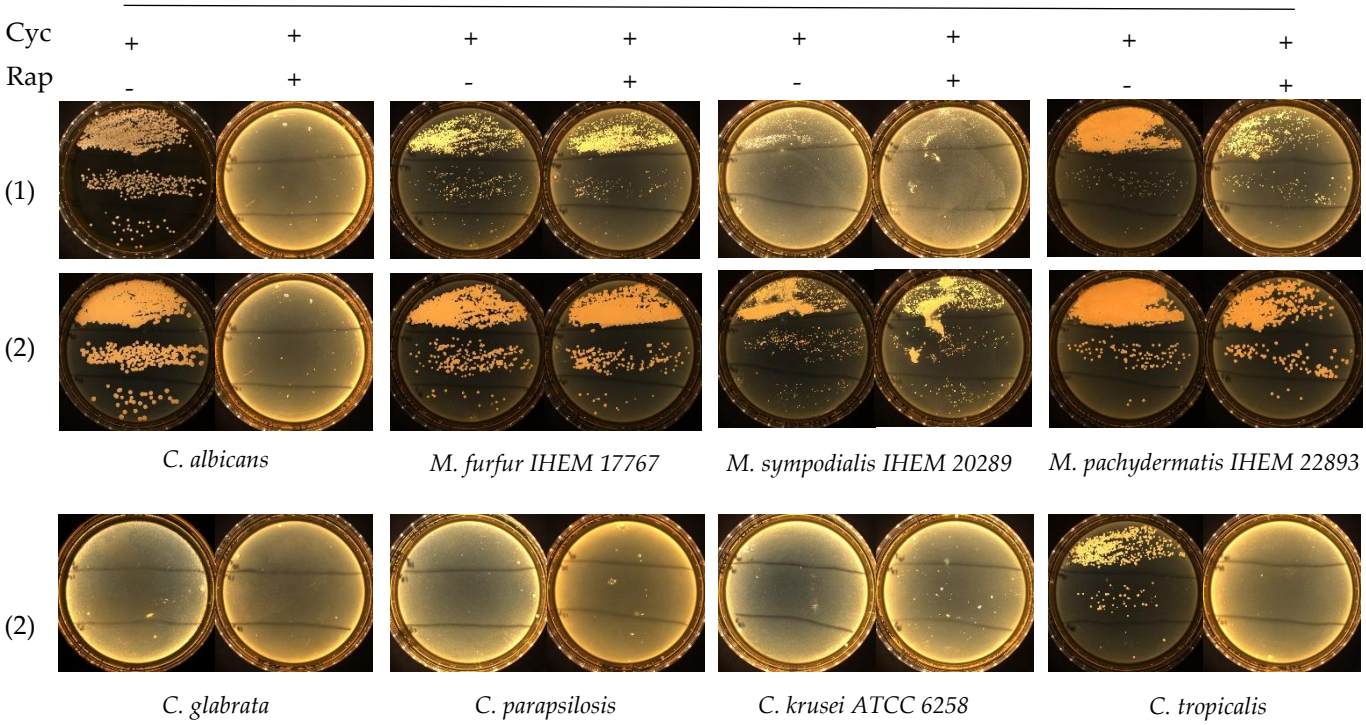

Figure 2. Growth testing via serial inocula dilution of *Candida* spp. and *Malassezia* spp. on the FastFung medium without (-) or with (+) 2 mg/L Rapamycin (Rap) and 500 mg/L Cycloheximide (Cyc). Culture of 48 h (1) and 72 h (2) of incubation.

4. Discussion

 doi:10.20944/preprints202107.0601.v1

The development of selective culture media is an important issue in medical mycology for isolation and culture of pathogens from clinical specimens. The use of antimicrobial agents remains the main strategy for the inhibition of undesirable microorganisms in culture. Here, we evaluated in vitro antifungal activity of rapamycin against M. furfur and *Candida* spp., and found that *M. furfur* is resistant with MIC values ≥ 32 mg/L (Table 1). However, rapamycin showed a higher activity against *Candida* spp. with MIC values ranging from 0.5 to 2 mg/L, except *C. krusei* ATCC 6258 (Table 1). This higher activity of rapamycin against *Candida* spp., especially *C. albicans* is consistent with the results reported in the literature [23,25,26]. MIC value ≥ 32 mg/L recorded for *C. krusei* ATCC 6258 deserves further investigations to determine whether rapamycin does not affect *C. krusei* species or whether our strain is resistant.

Growth testing on agar showed that cycloheximide and rapamycin have dual negative effects against *Candida* spp. in culture. Cycloheximide inhibited the growth of *C. glabrata*, *C. parapsilosis* and *C. krusei* ATCC 6258, whereas *C. albicans* and *C. tropicalis* were able to grow in FastFung medium supplemented with cycloheximide (Figure 2). The effects of cycloheximide against certain *Candida* spp. were already known [10,12,13]. By adding rapamycin, we were able to inhibit all candida spp. tested, as no growth were observed in culture (Figure 2). No effect of cycloheximide against Malassezia species were observed, which is in agreement with the literature [15,16].

One of the most striking findings was the resistance of Malassezia spp. against rapamycin. Both strains of M. furfur showed resistance with MIC value ≥ 32 mg/L (Table 1). These results were confirmed by testing M. furfur, M. sympodialis and M. pachydermatis in FastFung medium supplemented with rapamycin (Figure 2). Our findings are particularly interesting as rapamycin inhibit a broad-spectrum of fungi including filamentous fungi and yeasts [26]. These results shows that rapamycin can be a good candidate in selective agents. For example, it is well known that Candida spp. grow quickly whereas Malassezia spp. grow slowly. Rapamycin allows the growth of *Malassezia* spp., by limiting the growth of *Candida* spp. when sample with multiple fungal species are analysed. On the other hand, rapamycin can prevent contaminations during subculturing. Contamination problems are frequent in slow-growing fungi [30].

In conclusion, we propose the use of culture media such as FastFung supplemented with cycloheximide and antibiotics including rapamycin for isolation and culture of Malassezia species from polymicrobial samples. In the future, it will be interesting to test this selective medium with clinical specimens of varied origin including stools.

5. Conclusions

Based on our findings, we propose the use of culture media such as FastFung supplemented with cycloheximide and antibiotics including rapamycin for isolation and culture of *Malassezia* species from polymicrobial samples. In the future, it will be interesting to test this selective medium with clinical specimens of varied origin including stools.

Author Contributions: Abdourahim Abdillah: Conceptualization; Data curation; Investigation; Formal analysis; Methodology; Visualization; Writing the original draft. Stéphane Ranque: Conceptualization; Methodology; Investigation; Formal analysis; Resources; Supervision; Writing - review & editing.

Funding: This research was funded by the Institut Hospitalo-Universitaire (IHU) Méditerranée Infection, the French National Research Agency under the program "Investissements d'avenir", reference ANR-10-IAHU-03, the Région Provence Alpes Côte d'Azur and European funding FEDER PRIMI.

Acknowledgments: We thank Dirk Stubbe for graciously providing us with the *Malassezia* strains from the fungal collection BCCM/IHEM, Sciensano, Brussels, Belgium.

Conflicts of Interest: The authors declare no conflict of interests.

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