Optimal Culture Condition for Antifungal Susceptibility Tests of *Malassezia globosa*

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= Abstract =

Background: Although numerous culture conditions for *Malassezia* species were suggested, there were not so many objective evaluation articles in the literature.

Objectives: We examined the various culture conditions for *Malassezia globosa*.

Methods: *Malassezia globosa* culture conditions were assessed by Dixon's agar, modified Leeming-Notman medium in diverse oil content and temperature conditions.

Results: Maximum growth rate of *Malassezia globosa* was achieved at 3% olive oil. The optimal temperatures for the maximal growth of *M. globosa* were observed at 32~34℃.

Conclusion: In this study, we established the optimal culture condition for *M. globosa*, and confirmed its excellent utility for the antifungal susceptibility tests for *M. globosa* and *M. restricta*. Our results can help the investigators plan to do the prospective researches involving *Malassezia* species, such as the susceptibility test for newly developed antifungal agents. **[Kor J Med Mycol 2009; 14(4): 182-189]**

Key Words: *Malassezia*, Culture condition

INTRODUCTION

Malassezia species are normal flora of the human skin. They are major pathogenic fungi that cause the common skin disorders including pityriasis versicolor and *Malassezia folliculitis*,-seborrheic dermatitis, psoriasis, and atopic dermatitis in human[1~3]. Long chain fatty acids are essential for the growth of most *Malassezia* species they must be supplied from human skin lipids Lipid requirement results in the highest density of *Malassezia* in the sebaceous areas (such as the scalp), face, and upper trunk, and the lowest density on the hands. Due to their lipid requirement, *Malassezia* do not grow on a standard fungal culture medium, and should be grown on the specialized media supplemented with fatty acids such as Leeming-Notman agar, Dixon agar or Littman agar[4,5]. Another cause of difficult for *Malassezia* research in early days was the dimorphism of the species. Until 1977, many investigators believed that the yeast phase (denoted by *Pityrosporum*) and the mycelial phase (denoted by *Malassezia*) were different organisms. In 1977,

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three groups showed that these two phases could be interconverted due to the dimorphism of the organism. Therefore, the taxonomy of *Malassezia* have been somewhat confused, and it was not a long time ago that the systemic classification of *Malassezia* came to be possible. So far, analysis of morphological characteristics and physiological features, and the use of different Tween compounds, as well as molecular biological studies have allowed their classification into 13 species: *M. furfur*, *M. sympodialis*, *M. slooffiae*, *M. obtusa*, *M. globosa*, *M. restricta*, *M. pachydermatis*, *M. yamatoensis*, *M. nana*, *M. japonica*, *M. equine*, *M. caprae* and *M. dermatis*[6,7].

As for the optimal culture condition of *Malassezia* species, there have been only a few reports, partially due to the historical confusion of these fungi. Although olive oil has been most widely used for the isolation of lipophilic *Malassezia* species, other lipids can also be assimilated by *Malassezia*[8,9]. However, only a few authors have described which lipid classes can be metabolized by *Malassezia* species[10~13]. And, there have been no reports focusing on the comparison of the efficacy of each lipid on the growth of *Malassezia* species. Any information about the optimal temperature for the growth of *Malassezia* has not been reported. In 1987, Leeming et al. reported that *M. furfur* showed the highest growth rate at 34℃ among the several temperatures from 27℃ to 37℃[14]. Since Leeming's, there has been no reports focusing on the optimal temperature for *Malassezia* species. The optimization of culture condition is necessary and should be established for the successful studies about *Malassezia*, including antifungal susceptibility tests.

Several antifungal agents have been shown to be effective for the treatment of *Malassezia* species. Especially, ketoconazole and albaconazole have shown the best *in vitro* activity against all species tested, and itraconazole has generally good activity[15]. But, the optimization of the culture condition is the prerequisite for the study of antifungal susceptibility. In 1997, the National Committee for Clinical Laboratory Standards approved a broth micro- and macrodilution method for susceptibility testing of yeasts with RPMI 1640 medium (NCCLS-M27A)[16]. Alternative methods have been assessed with the broth microdilution method using media such as Leeming-Notman and modified Dixon[17]. These media are turbid,-therefore,-and the limitation is that the visual and turbidimetric results are difficult to interpret[18,19]. Plate methods, plate disk methods, and tube methods are useful for microbial sensitivity examinations. However, they are all labor-intensive and require great efforts. In particular, fungi require a long period of time because the growth rate is slow. Therefore, the methods mentioned cannot be widely used for the susceptibility tests for fungi in daily practice.

The aim of this study was to determine the optimal culture condition for *Malassezia* species, such as adequate temperature and the additive oil components and the oil concentrations. In addition, using this optimal culture condition, we evaluated the antifungal activity of six antifungal agents (ketoconazole, fluconazole, itraconazole, ciclopirox, selenium sulfide, and zinc pyrithione) against *M. globosa* and *M. restricta*, by measuring minimal inhibitory concentrations (MIC) with a broth macro-dilution method (EUCAST Definitive Document EDef 7.1)[19].

MATERIALS AND METHODS

1. *Malassezia* strains and Chemicals

M. globosa and *M. restricta* strains were pur-

chased from The American Type Culture Collection (ATCC, Manassas, VA). Ketoconazole, fluconazole, itraconazole, ciclopirox, and selenium sulfide were purchased from Sigma (St. Louis, MO) or Aldrich Chemical Co. (Milwaukee, WI). Other chemicals were of the highest grade commercially available.

2. Determination of the optimal oil and temperature condition

For broth assays, inocula were prepared by the culture of *M. globosa* on modified Dixon's agar for five to seven days. Then, the colonies were transferred on modified Leeming-Notman medium containing 0.1% glucose, 0.1% peptone, 0.8% bile salts, 0.2% yeast extract, 0.1% glycerol, 0.5% Tween 60, 3% olive oil, and 50 μg/ml chloramphenicol[13]. The inoculua suspensions were prepared at the final concentration of $(1.5\pm1.0)\times10^5$ cells/ml adjusted with a UV/visible spectrophotometer (Shimazu, Japan). All inocula suspensions were vortexed for 20 s to disperse *Malassezia* clumps. One of three kinds of oils, olive oils, soybean oil, and canola oil, was added in each suspension, at the various oil concentrations from 1.5% to 6%. The temperature condition was modified variously at 24, 28, 30, 32, 34, and 37℃. The growth of fungi under the different oils and temperature conditions was monitored every 24 h for eight days of incubation. For the temperature condition and antifungal susceptibility tests, the media mixed with 3% olive oil was used.

3. Antifungal agents

Ketoconazole, fluconazole, itraconazole, ciclopirox, selenium sulfide, and zinc pyrithione were tested for their antifungal activity. Ketoconazole, itraconazole, ciclopirox and selenium sulfide were dissolved in dimethyl sulfoxide, and fluconazole and zinc pyrithione in distilled water. All stock solutions were prepared at the concentration of 16 μg/ml and serially diluted to 0.03 μg/ml with RPMI 1640 broth (DIBCO, NY, USA). The solutions were sealed and stored frozen at -80℃ until used.

4. Susceptibility test medium

For the susceptibility testing, RPMI 1640 broth (without bicarbonate) supplemented with L-glutamine and 2% glucose was used after buffering with 3-N-morpholino-propanesulfonic acid to pH 7.0.

5. Susceptibility testing

The broth macrodilution method was performed in accordance with the EUCAST guidelines in document EDef 7.1 using Modified dixon's agar. The yeast colonies were suspended in sterile water in polystyrene plastic tubes, and the turbidity of the resulting suspensions was adjusted to a calibrated 0.5 McFarland at a 530 nm wavelength with a spectrophotometer. The final concentrations (15.6 ng/ml ~ 8 μg/ml) of the antifungal agents in the suspensions were adjusted.

The growth of fungi at 32℃ was monitored at 530 nm, every 24 h for five days. The growth of antifungals-treated isolates was compared with the growth of a antifungal-free control. MIC was determined as the lowest concentration of the antifungal agents that produced 50% growth in comparison with the control. Each isolate was tested six times on separate occasions.

6. Quality control

One EUCAST quality control strain, *Aspergilus fumigatus* was included on every testing day to check the accuracy of the drug dilutions and the reproducibility of the results.

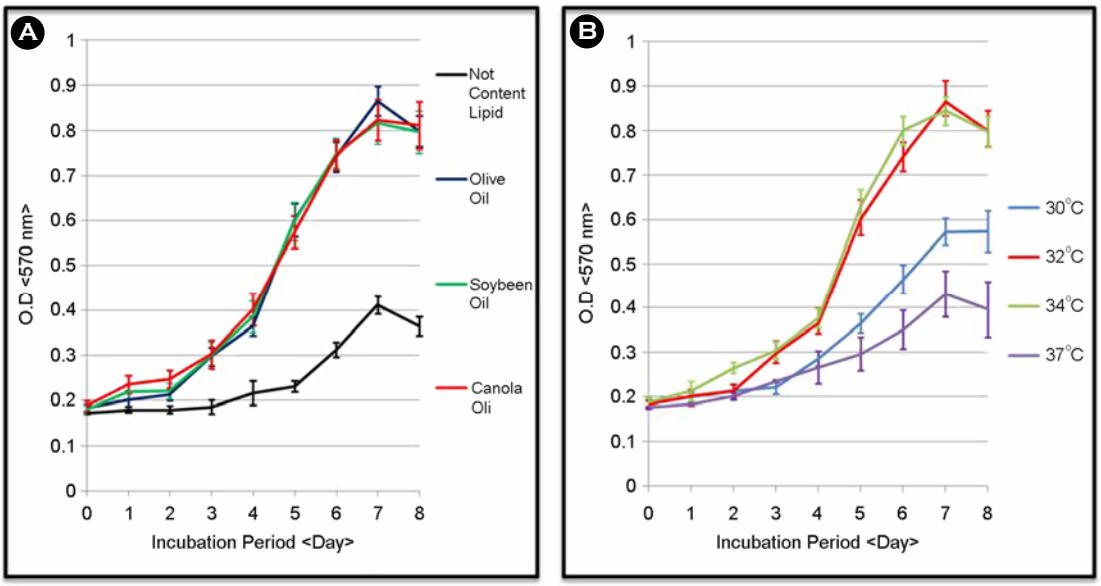

Fig. 1. Growth curve of *M. globos*a at various lipids and temperatures.

RESULTS

1. The optimal lipid and temperature condition for *Malassezia* species

M. globosa was cultured under the various lipid and temperature conditions using the modified Leeming-Notman medium. Three kinds of lipids (olive oil, soybean oil, and canola oil) were added in the culture medium, and the effects on the growth of *M. globosa* were evaluated. Lipid composition did not show the significant differences of the growth rate of *M. globosa* in the culture media (Fig. 1). Regardless of the lipids used, the maximal growth in all groups was observed after seven days of incubation (Fig. 1A). The optimal temperatures for the maximal growth of *M. globosa* were observed at 32~34℃ (Fig. 1B).

Concentration effects of lipid for the growth of *M. globosa* were evaluated using from 1.5% to 6.0 % olive oil, to determine the optimal lipid concentration. The maximal growth of *M. globosa* was observed at 3.0% olive oil. The high lipid concentration of more 4.5% showed more or less the inhibitory effect on the growth (Fig. 2).

2. Susceptibility of *M. globos*a and *M. restricta* against six antifungal agents

RPMI 1640 was chosen as a susceptibility test medium because it allowed good growth of matured form of *Malassezia* species colonies appeared after 96 h post inoculation. Therefore, the MIC values of *M. globosa* and *M. restricta* could be determined after at least 96 h. The ranges of MIC and MIC_{50} (MIC for 50%) of *Malassezia* isolates, were determined by macrodilution method for six drugs (Table 1). The MIC ranges for *Aspergillus fumigatus* KTCC 6415 as a reference strain were within the values standardized by EUCAST Definitive Document EDef 7.1[19]. The lowest MIC_{50} values for both of *M. globosa* and *M. restricta* were observed in ketoconazole while selenium sulfide and zinc pyrithione showed relatively high concentration.

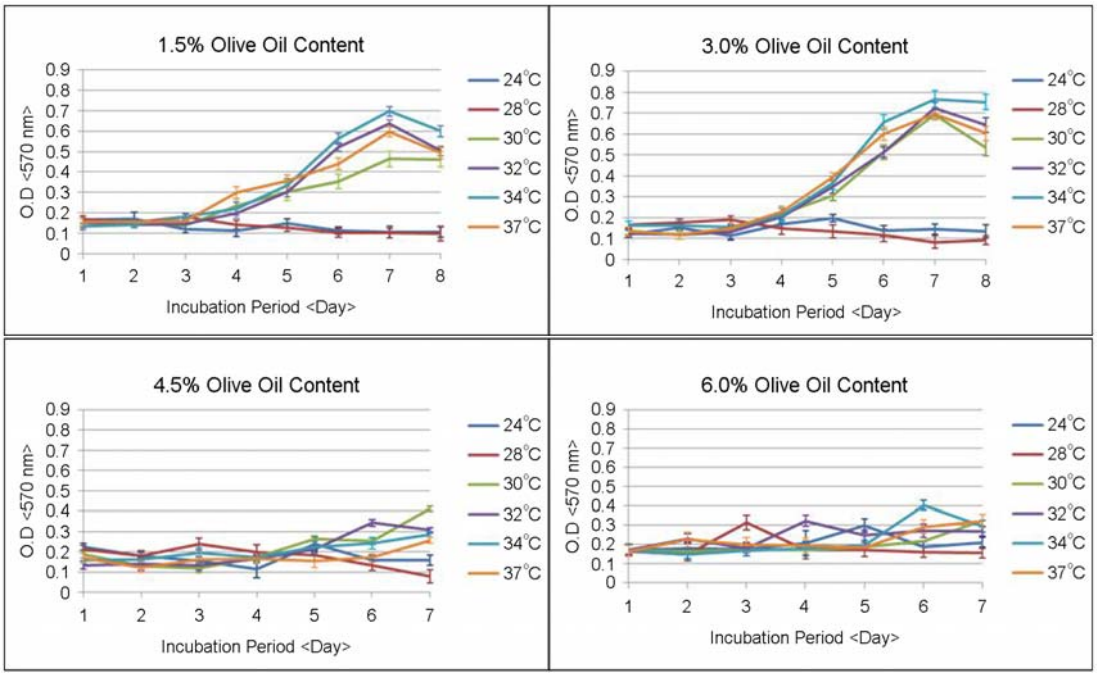

Fig. 2. Growth curve of *M. globosa* at the various olive oil concentrations.

Table 1. The MIC ranges and MIC_{50} of six antifungal agents by macrodilution method

Species/Antifungal	MIC (μg/ml)	
	Range	MIC_{50}
M. globosa		
Ketoconazole	0.03~0.125	0.03
Fluconazole	0.06~1	0.125
Itraconazole	0.03~0.125	0.03
Ciclopirox	0.25~1	0.5
Selenium sulfide	1~8	2
Zinc pyrithion	0.5~8	1
M. restricta		
Ketoconazole	0.03~0.125	0.06
Fluconazole	0.06~1	0.25
Itraconazole	0.03~0.125	0.03
Ciclopirox	0.25~2	0.5
Selenium sulfide	1~8	2
Zinc pyrithione	1~8	1

DISCUSSION

The genus *Malassezia* is normal skin flora of humans and other warm-blooded animals. *Malassezia* species have been associated with dandruff, an easily recognizable skin flaking condition occurring in 30~95% of general population. Despite their widespread occurrence and association with multiple common skin disorders, remarkably little is known about these fungi[20]. There have been only a few reports focusing on the optimal culture condition of *Malassezia* species. Lipid is one of the most important factors which should be considered in the culture of lipophilic *Malassezia* species. Olive oil has been the most widely used lipid for the culture of *Malassezia* species, and other lipids can also be assimilated by *Malassezia*. Mayser *et al*. reported that castor oil, containing ricinoleic acid, promoted the growth of *M. globosa*

only, whereas the other several lipids promoted the growth of six species of *Malassezia* including *M. globosa*[8]. Even the lipids included in the topical dermatologic agents were shown to promote the growth of *M. furfur* and *M. sympodialis*[9]. However, previous reports showed only the possibility of the growth according to the kind of lipid used. There have been no reports focusing on the detailed comparison of the efficacy of each lipid on the growth of *Malassezia* species, to the best of our knowledge[8,9]. Our results showed the similar effects of three kinds of lipids, olive oil, soybean oil and canola oil for the growth of *M. globosa*. Three lipids also showed the similar results regarding the efficacy of each concentration, showing the best growth at 3% concentration. Olive oil is composed mainly of the mixed triglyceride esters of oleic acid and palmitic acid. Soybean oil contains alpha-linolenic acid and isoflavones such as genistein and daidzein. Canola oil is composed mostly of olieic acid, linoleic acid, and alpha-linolenic acid. This similarity of the constituents of three lipids may have resulted in the similar efficacy for the growth of *M. globosa*. As for the incubation temperature, there have been almost no reports studying the optimal temperature for the growth of *Malassezia* species after Leeming *et al*. reported 34℃ as the best temperature for the growth of *M. furfur*[14]. Our results showed that *M. globosa* exhibited the highest growth rate at 32~34℃, and seven days-incubation time was required to obtain full growth.

We evaluated the antifungal activity of six antifungal agents against *M. globosa* and *M. restricta*, under the most optimal culture condition which we found. The incubation media was mixed with 3% olive oil, and incubated at 32℃. The MIC values were determined in five days for *M. globosa* and *M. restricta*, because this duration time was required to obtain good growth with these species. Our results showed generally good accordance with the previous results[15,21,22]. The MIC ranges of *M. globosa* and *M. restricta*, were 0.03 to 0.125 μg/ml for ketoconazole, 0.06 to 1.0 μg/ml for fluconazole, 0.03 to 0.125 μg/ml for itraconazole. MIC of fluconazole was higher than other azoles. These data are similar to those found by Xisto *et al*[21]. The MICs of ciclopirox ranges were 0.25 to 2.0 μg/ml, somewhat lower than previous report[22]. The MIC ranges for selenium sulfide and zinc pyrithione were 1.0 to 8.0 μg/ml, and MIC_{50} of selenium sulfide was higher than that of zinc pyrithione. Previously, the MIC of selenium sulfide for *Malassezia* species has been reported only once. However, in that report, the target species was designated as '*Pityrosporum ovale*', now recognized as *M. slooffiae*, *M. obtus*e or *M.*

Table 2. Comparison of the previously reported MIC ranges with ours for six antifungal agents[15,22,23]

Species/ Antifungal	Our Results	Previous Reports
	MIC Range (μg/ml)	MIC Range (μg/ml)
M. globosa		
Ketoconazole	0.03~0.125	0.008~0.125
Fluconazole	0.06~1	0.125~64
Itraconazole	0.03~0.125	0.016~6.3
Ciclopirox	0.25~1	7.3
Selenium sulfide	1~8	ND
Zinc pyrithione	0.5~8	0.3
M. restricta		
Ketoconazole	0.03~0.125	0.016_0.626
Fluconazole	0.06~1	0.5_1
Itraconazole	0.03~0.125	0.0156_6.3
Ciclopirox	0.25~2	7.3
Selenium sulfide	1~8	ND
Zinc pyrithione	1~8	0.15

ND = no data

sympodialis[23]. Therefore, our study is the first to clarify the MIC of selenium sulfide for *M. globosa* and *M. restricta*. Comparison of the previous results with ours is summarized in Table 2[15,22,23].

In this study, we established the optimal culture condition for *M. globosa*, and confirmed its excellent utility for the antifungal susceptibility tests for *M. globosa* and *M. restricta*. Our results can help the investigators plan to do the prospective researches involving *Malassezia* species, such as the susceptibility test for newly developed antifungal agents.

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