Malassezia folliculitis is caused by cutaneous resident Malassezia species

Malassezia folliculitis [MF] is caused by the invasion of hair follicles by large numbers of Malassezia cells, but it remains unclear which Malassezia species are involved in the disease. To clarify this situation, Malassezia species isolated from lesions of MF patients were analyzed by both culture and non-culture methods. In addition, Malassezia species recovered from the non-lesion areas of the skin of MF patients and skin samples of healthy subjects were included in this study. The test population consisted of 32 MF patients and 40 healthy individuals. The lesions were obtained using a comedone extractor, while swabs were employed to obtain skin samples from non-lesion areas of the patients and healthy subjects. Malassezia DNA was analyzed using a real-time PCR technique. The detection limit of the culture method was 5 CFU/cm² as opposed 50 cells/cm² with non-culture procedures. The predominant species recovered from MF lesions were M. globosa and M. sympodialis by culture method analysis, and M. restricta, M. globosa, and M. sympodialis with non-culture methods. These results were in agreement with those found with samples from non-lesion skin areas of MF patients and healthy subjects. This study clarified that MF is caused by Malassezia species that are part of the cutaneous microflora and not by exogenous species.

Keywords Malassezia, Malassezia folliculitis, human cutaneous resident microbiota, real-time PCR

Introduction

Members of the genus Malassezia are part of the normal microbiota of human skin. The taxonomy of this genus was recently revised to include the following seven species; M. furfur, M. globosa, M. obtusa, M. restricta, M. pachydermatis, M. slooffiae, and M. sympodialis [1–3]. Furthermore, this reevaluation included the splitting of M. furfur into five species; M. furfur, M. globosa, M. obtusa, M. restricta, and M. slooffiae. In addition, six new species, M. dermatis, M. japonica, M. yamatoensis, M. nana, M. caprae, and M. equina were recently proposed [4–8].

Malassezia species are associated with Malassezia folliculitis [MF], pityriasis versicolor, seborrheic dermatitis, dandruff, atopic dermatitis, and psoriasis [9]. MF is caused by the invasion of large numbers of Malassezia cells into hair follicles [10]. This results in the development of asymptomatic or pruritic erythematous papules and sometimes pustules on the back, chest, and upper aspects of the arms [10,11]. MF was called Pityrosporum folliculitis before the 1980s and was assumed to be caused by Pityrosporum ovale [12]. However, with the incorporation of Pityrosporum spp. into the genus Malassezia, it still remains unclear which of the Malassezia species are involved in MF.
The identification of *Malassezia* species may be accomplished by either cultural [13–15] or non-culture methods [16–22]. The latter would appear to be superior since the species characteristics are difficult to differentiate in culture. On the other hand, the culture methods are more helpful in dealing with living cells. There have been few comparative studies using both culture and non-culture methods in the analysis of the *Malassezia* components of the normal skin microbiota. The aim of this investigation was to clarify the *Malassezia* species that cause MF by identifying the members of the genus recovered from lesions by both the culture and non-culture methods. Moreover, the *Malassezia* species isolated from non-lesion areas of the skin of MF patients and those from healthy subjects were identified and compared.

**Materials and methods**

**Subjects**

The subjects were 32 Japanese outpatients with MF (17 males and 15 females, with an average age of 31 ± 11) who were seen at Fujita Health University Hospital (from June to September 2007) and Kansai Medical University Kori Hospital (from May to September 2005). The diagnosis of MF was based on the observations of erythematous papules and pustules by dermatologists and the finding of high levels of yeasts. Lesions were noted on the back in 20 patients, upper chest with nine and the neck with the remaining three individuals. While clinical samples were obtained from all subjects for culture analysis, 25 of these same individuals (13 males and 12 females with an average age of 33 ± 11 having lesions on the upper back – 15, upper chest – 7, and neck – 3) provided specimens for non-culture methods of study. As a comparison, samples from 40 healthy Japanese subjects (20 males and 20 females with an average age of 33 ± 7) were analyzed from June to September 2007.

**Sample collection**

Papules and pustules were obtained from the lesions of patients using a comedone extractor. In addition, 10 cm² skin samples were collected by the swab method using 5 ml of phosphate buffer containing 2% polysorbate 80 (pH 7.0).

For culture studies, 0.1 ml of the collected samples were incubated at 32°C on Leeming & Notman agar medium (LNA) for 14 days [21]. Colonies found on initial cultures were then sub-cultured for purification on LNA for 2 to 7 days prior to extraction of *Malassezia* DNA. The non-culture method consisted of the extraction of *Malassezia* DNA directly from 3 ml of the collected samples.

**DNA extraction**

The collected samples were placed in lysing solution (100 mM Tris-Cl, 10 mM EDTA, 0.5% sodium dodecyl sulfate, pH 8.0) and incubated for 15 min at 100°C [16], after which the suspension was extracted with phenol-chloroform-isoamyl alcohol (25:24:1). Subsequently, the samples were extracted with chloroform-isoamyl alcohol (24:1) and DNA was precipitated with 2-propanol, using Dr genTLE precipitation carrier (Takara, Shiga, Japan). The DNA pellet was resuspended in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0).

**Detection of Malassezia DNA**

*Malassezia* spp. DNA in the samples were analyzed using a real-time PCR system (7300 Real Time PCR system; Applied Biosystems, Foster City, CA, USA) and a quantitative PCR reagent (Platinum SYBR Green qPCR SuperMix-UDG with ROX; Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The primer sets used are shown in Table 1. Nine *Malassezia* species isolated from human skin were targeted in this study.

The primers were designed using the Genbank database along with primer-designing software ‘Primer 3’ and ‘Primer express (Applied Biosystems)’ or pre-

<table>
<thead>
<tr>
<th>Oligonucleotides used in the detection of Malassezia DNA</th>
<th>Sequence (5’ → 3’)</th>
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<tbody>
<tr>
<td><strong>Species</strong></td>
<td><strong>Sequence</strong></td>
</tr>
<tr>
<td>M. globosa</td>
<td>GGCCTCTCGGCCTCTCTCT</td>
</tr>
<tr>
<td></td>
<td>CACCAACAGATCTCCTACAG</td>
</tr>
<tr>
<td>M. sympodialis</td>
<td>GACCCTCGCTACCGCTCTCT</td>
</tr>
<tr>
<td></td>
<td>GACCACACAGCAATGACAC</td>
</tr>
<tr>
<td>M. restricta</td>
<td>TGCCATGAAATTCGCCCAA</td>
</tr>
<tr>
<td></td>
<td>AGGCAACCCATCCAGACCACCAT</td>
</tr>
<tr>
<td>M. dermatis</td>
<td>GCCCTGATGCTCGTGGTTAT</td>
</tr>
<tr>
<td></td>
<td>CTTGTCGCGACGACTCA</td>
</tr>
<tr>
<td>M. furfur</td>
<td>CCGGAGGTTGCGGATT</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
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<td>GGTTCGCCCACTCGTTAGAC</td>
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<tr>
<td></td>
<td>GCACACAGCAATGAC</td>
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<td>M. slooffiae</td>
<td>ATCCACATTCTCCACCAAA</td>
</tr>
<tr>
<td></td>
<td>CGGACGCCCATTAGCA</td>
</tr>
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<td>M. yamatoensis</td>
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<td></td>
<td>GGCATGGCCCATCCAA</td>
</tr>
</tbody>
</table>
vious papers on *M. globosa* [19] and *M. japonica* [5]. The specificity of the primers was investigated using ‘NCBI BLAST’ and confirmed by amplification and melting temperatures using standard strains of Malassezia spp.

The standard curves of each Malassezia spp. comprising the Malassezia microbiota were generated using quantitative analysis of the skin surface with *M. globosa* CBS 7966, *M. restricta* CBS 7877, *M. sympodialis* CBS 7222, *M. furfur* NBRC 0656, *M. obtusa* CBS 7876, *M. slooffiae* CBS 7956, *M. dermatis* JCM 11348, *M. japonica* JCM 11963, and *M. yamatoensis* CBS 9725 counted beforehand using a bacteria counting chamber. The dispersion of Malassezia cells of each species was used after stirring sufficiently to decrease clumping as much as possible.

**Statistical analysis**

For the quantitative analysis, the numbers of Malassezia species less than the detection limit (the culture method, 5 CFU/cm²; the non-culture method, 50 cells/cm²) were assumed to be 10⁰/cm². Student’s t-test was used for statistical analysis in this study.

**Results**

**Standard curves for Malassezia analysis by the non-culture method**

Standard curves for each Malassezia spp. were plotted for each primer set using cycle threshold (Ct) values obtained from known numbers of Malassezia cells. Each species-specific primer set amplified only the targeted Malassezia species at the specific melting temperature (Tm value). Examples of the standard curves are shown in Fig. 1 for of *M. globosa*, *M. restricta*, *M. sympodialis*, *M. furfur*, and *M. dermatis* (the standard curves of *M. obtusa*, *M. slooffiae*, *M. japonica*, and *M. yamatoensis* are not shown). A fixed quantity of over than 500 cells was identified in all Malassezia species, resulting in the detection limit of Malassezia DNA at 500 cells (50 cells/cm²) in this study.

**Analysis by the culture method**

The number of Malassezia species detected in lesions of MF, non-lesion areas of MF patients, and the skin of healthy subjects is shown in Fig. 2a. Samples from MF lesions contained 1.7 ± 0.7 (mean ± SD) species, compared with 1.5 ± 0.9 species in samples from the non-lesion skin of MF patients and 1.9 ± 0.8 species in samples from the healthy subjects. There were no significant differences in the values (*P > 0.05*).

Fig. 3a shows the detection rates of *Malassezia* species in MF lesions, the non-lesion skin of MF patients, and the skin of healthy subjects. The species detected in the lesions and non-lesion skin of MF patients were *M. globosa*, *M. sympodialis*, *M. restricta*, *M. dermatis*, and *M. furfur*. In addition, *M. obtusa* was detected at a low level in healthy subjects. In contrast, *M. slooffiae*, *M. japonica*, and *M. yamatoensis* were not detected in any of the samples in this study. The predominant species in MF lesions were *M. globosa* (72%) and *M. sympodialis* (52%). These Malassezia species were also detected in the non-lesion skin of MF patients (*M. globosa*, 83%; *M. sympodialis*, 35%) and the healthy subjects (*M. globosa*, 79%; *M. sympodialis*, 60%).

Fig. 4a shows the numbers of each Malassezia species detected in the non-lesion skin of MF patients and the healthy subjects. The total number of Malassezia cells was scored as 2.7 ± 0.87 (mean ± SD) CFU/cm² in MF patients and 2.8 ± 0.84 CFU/cm² in the healthy subjects. There was no significant difference between MF patients and the healthy subjects with respect to the total number of Malassezia cells and each Malassezia species detected in the samples (*P > 0.05*).}

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cm² in MF patients and 2.21 ± 1.53 CFU/cm² in the healthy subjects. There was no significant difference between MF patients and the healthy subjects with respect to both the total number of Malassezia cells and each Malassezia species detected in the samples (P > 0.05).

There were no significant differences between the results obtained with samples from the upper chest and back (and neck in MF lesions) by the culture and the non-culture methods (data not shown). Moreover, there were no significant differences in the results of samples provided from patients of Fujita Health University Hospital and those of Kansai Medical University Kori Hospital (data not shown).

Discussion

This study demonstrated that M. globosa, M. sympodialis, M. restricta, M. dermatis, and M. furfur are found in MF lesions. The predominant species were M. globosa and M. sympodialis by the culture method analysis and M. restricta, M. globosa, and M. sympodialis through the use of non-culture method analysis. The number of species of Malassezia detected in MF

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lesions was occasionally two or more. These results agreed with those obtained with skin samples from healthy subjects, namely these same species are part of the normal human cutaneous resident microbiota. Moreover, the composition of the *Malassezia* microbiota on non-lesion skin of MF patients was the same as that found with normal human skin. These results suggested that MF is caused by cutaneous resident *Malassezia* species and not by specific exogenous *Malassezia* species.

The *Malassezia* cutaneous microbiota of healthy subjects has been analyzed previously. Using culture methods, *M. globosa* and *M. sympodialis* have been reported as the predominant species on the skin of the trunk in healthy subjects [13,14]. On the other hand, *M. restricta* was also found as a predominant species in cases studied by non-culture methods [16,21,22]. The present results agreed with those of previous reports in the literature. In particular, each sample in this investigation was analyzed by non-culture and culture methods at the same time. Our results suggest that *M. restricta* cannot be counted precisely by the culture method using Leeming & Notman agar medium [14,15] or Dixon agar medium [13] due to non-cultivable types included in *M. restricta*. 

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Fig. 2 Numbers of species of *Malassezia* yeasts detected on MF patients and healthy subjects. The numbers of the species of *Malassezia* yeasts detected by the culture method (a) and the non-culture method (b).

Fig. 3 Detection rates of *Malassezia* spp. on MF patients and healthy subjects. Detection rates of *Malassezia* spp. by the culture method (a) and the non-culture method (b). gl, *M. globosa*; sy, *M. sympodialis*; re, *M. restricta*; de, *M. dermatis*; fu, *M. furfur*; ob, *M. obtusa*; slo, *M. slooffiae*, *M. japonica*, and *M. yamatoensis* were not detected in any of the samples.

Fig. 4 Numbers of each *Malassezia* species detected on non-lesion skin. The numbers of each *Malassezia* species detected by the culture method (a) and the non-culture method (b).

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For the quantitative analysis in this study, the total number of *Malassezia* species identified by the non-culture method showed few differences with those obtained by the culture method. This phenomenon is dependent on differences in the detection limits, that is, the detection limit of the culture method is 5 CFU/cm² and that of the non-culture method is 50 cells/cm², although less than the detection limit was analyzed as 10³ cells/cm² in the statistical analysis. Improvement of the detection limit of the non-culture method may be necessary for further quantitative studies of *Malassezia* species. On the other hand, the fact that the total number of *Malassezia* cells on the skin analyzed by the culture method and that by the non-culture method did not differ particular may suggest that almost all *Malassezia* species on the skin are viable cells.

The exact mechanism involved in MF is still unknown. MF is marked by the presence of an inflammatory infiltrate consisting of lymphocytes, histiocytes, and neutrophils, along with focal rupture of the follicular epithelium [11,24,25]. Faergemann et al. [26] reported that an irritant non-immunogenic stimulant of the immune system is important in the inflammation. The inflammatory reaction may be a result, in part, of the ability of a *Malassezia* lipase to hydrolyze triglycerides into free fatty acid [25–28]. It was also reported that *Malassezia* suppressed the production of the inflammatory cytokines in human peripheral blood mononuclear cells *in vitro* [29,30]. On the other hand, *Malassezia* species induce the production of inflammatory cytokines on human epidermal keratinocytes via Toll-like receptor 2 *in vitro* [31]. Moreover, Watanabe et al. [32] also reported that there are differences in the ability to induce cytokine production on keratinocytes among *Malassezia* species. The results in this study indicate that MF is induced by the overgrowth of cutaneous resident *Malassezia* species by an abnormal differentiation of hair follicles. Hill et al. [33] also noted that the overgrowth of *Malassezia* yeasts is a secondary occurrence caused by the occlusion of the follicle. For clarification of the mechanisms involved in MF, it will be necessary to conduct additional investigation to study the relationship between each cutaneous resident *Malassezia* species and pathogenesis. Moreover, the *Malassezia* species detected in MF lesions were not quantified in this study because of sampling problems. The quantitative analysis of *Malassezia* species in the lesions might have to be executed in the future.

**Declaration of interest:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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**References**


This paper was first published online on iFirst on 10 August 2009.


