

# Quantitative Analysis of Cutaneous *Malassezia* in Atopic Dermatitis Patients Using Real-Time PCR

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**Abstract:** We quantified the cutaneous *Malassezia* in patients with atopic dermatitis using a real-time PCR assay. Seven to 12 times more *Malassezia* colonized the head and neck compared to the trunk or limbs, and the species *M. globosa* and *M. restricta* accounted for approximately 80% of all *Malassezia* colonization at any body site.

**Key words:** Atopic dermatitis, *Malassezia*, Real-time PCR

The basidiomycetous yeasts, *Malassezia* require lipids for growth, and colonize the skin of humans and animals. These microorganisms are thought to be the causative agents of seborrheic dermatitis and pityriasis versicolor (3). During the last decade, the relationship between *Malassezia* in the cutaneous flora and the exacerbation of atopic dermatitis (AD) has been investigated (2, 4, 9). Anti-*Malassezia* IgE antibody is detected in patients with AD, but not in healthy subjects. In particular, in head and neck AD, a significant correlation exists between the level of anti-*Malassezia* IgE and clinical severity (1). Treatment with antifungal agents, such as ketoconazole and itraconazole, leads to clinical improvement in head and neck AD with a decrease in *Malassezia* colonization (7). These results suggest that cutaneous *Malassezia* is one of the factors exacerbating AD. Currently, the genus *Malassezia* consists of 11 species: *M. dermatis*, *M. furfur*, *M. globosa*, *M. japonica*, *M. nana*, *M. obtusa*, *M. pachydermatis*, *M. restricta*, *M. slooffiae*, *M. sympodialis*, and *M. yamatoensis*. *Malassezia nana* and *M. pachydermatis* show affinity to animals, while the remaining nine species colonize human skin. Several studies have examined which species are involved in exacerbating AD. Sugita et al. (9) developed a direct detection assay of *Malassezia* based on nested PCR as a non-culture method, and analyzed cutaneous *Malassezia* flora of the skin in AD patients. *M. globosa* and *M. restricta* were detected in

almost all AD patients, while other *Malassezia* species were detected in fewer than 40% of cases. However, the accurate and reliable distribution of *Malassezia* at each body site has not yet been determined. In this study, we quantified *Malassezia* of the skin in AD patients at various body sites using a real-time PCR assay with a *Taq-Man* probe as a non-culture method.

Thirty-four AD outpatients at Tokyo Medical University Hospital participated in the study (17 males and 17 females; age,  $34.0 \pm 10.3$  years). Written informed consent was obtained from each patient. Disease severity was minor in 12 cases, moderate in 18 cases, and severe in four cases. No patient received any antifungal therapy.

OpSite<sup>TM</sup> transparent dressings (Smith and Nephew Medical, Hull, U.K.) were applied to the lesional skin of AD patients. Samples were collected from skin lesions on the head and neck, limbs, and trunk. The OpSite dressing (3×3 cm) was placed in a 1.5-ml Eppendorf tube with 1 ml of lysing solution (100 mM Tris-HCl [pH 8.0], 30 mM EDTA [pH 8.0], and 0.5% sodium dodecyl sulfate) and incubated for 15 min at 100 °C. The suspension was transferred to a new tube, and was extracted with phenol–chloroform–isoamyl alcohol (25:24:1, vol/vol/vol). Subsequently, the samples were extracted with chloroform–isoamyl alcohol (24:1, vol/vol) and the DNA was precipitated with 2.5 volumes of ethanol with 3 M sodium acetate and Ethaminate (Nippon Gene, Toyama, Japan) as a precipitation

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Abbreviations: AD, atopic dermatitis; ITS, internal transcribed spacer.

activator according to the manufacturer's instructions. The DNA pellet was resuspended in 30 µl of TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]) and stored at -20 °C until use. An unused OpSite™ dressing was used as a negative control.

Three sets of oligonucleotide primer and *TaqMan* probe pairs were designed for detecting all *Malassezia* species, *M. globosa* only, and *M. restricta* only. The first sequence was designed from the conserved region of the *Malassezia* 26S rDNA sequence, and the two latter nucleotide sequences were designed from the internal transcribed spacer (ITS) 2 region of the rRNA gene (Table 1). The real-time PCR assay was quantified using serial dilutions of *Malassezia* plasmid DNA. The 26S rDNA and ITS regions of all 11 currently accepted *Malassezia* species were amplified by PCR with the primers pITS (GTCGTAACAAGGTTAACCTGCGG) and NL4 GGTCCGTGTTTCAAGACGG) (5, 8). The PCR products were inserted into the plasmid vector pCR 2.1 (Invitrogen, Carlsbad, Calif., U.S.A.) according to the manufacturer's instructions. The plasmid was extracted from an overnight culture of *Escherichia coli* using QIAprep (QIAGEN, Santa Clarita, Calif., U.S.A.). The plasmid DNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Del., U.S.A.). To test sensitivity, tenfold serial dilutions were made in triplicate, ranging from 10<sup>1</sup> to 10<sup>9</sup> copies. These serial dilutions were used to derive a standard curve for quantification. For real-time PCR, 50 µl of a PCR mixture (Applied Biosystems, Foster, Calif., U.S.A.), 200 nM of each primer, and 250 nM *TaqMan* probe were placed in each well of a 96-well plate. Amplification and detection were performed with the ABI PRISM 7500 sequence detection system (Applied Biosystems) with the following cycle parameters: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. Standard curves for each organism were plotted for

each primer-probe set using the cycle threshold (*Ct*) (the cycle number at which the threshold fluorescence was reached) values obtained by amplifying successive tenfold dilutions of a known concentration of plasmid DNA. The Mala-F, Mala-R, and Mala-MGB primer/probe set was capable of detecting plasmid DNA derived from the 11 *Malassezia* species linearly for dilutions from 10<sup>2</sup> to 10<sup>9</sup> copies (Fig. 1). The sensitivity of the linearity of the sets was the same for all 11 *Malassezia* species. Therefore, we used the plasmid derived from *M. globosa* to quantify all *Malassezia* species. The *M. globosa*-specific and *M. restricta*-specific primer/probe sets could also detect the plasmid DNA derived from *M. globosa* or *M. restricta* linearly for dilutions from 10<sup>2</sup> to 10<sup>9</sup> copies (Fig. 1). Each species-specific primer/probe set amplified the targeted species only, and no other *Malassezia* species.

The cross-reactivity of the assay against cutaneous microorganisms was further assessed using DNA extracted from the following species: *Candida parapsilosis*, *C. guilliermondii*, *Cryptococcus diffluens*, *C. liquefaciens*, *Rhodotorula mucilaginosa*, *Staphylococcus aureus*, *S. epidermidis*, and *Micrococcus luteus*. The assay did not cross-react with any of these organisms.

Using the real-time PCR assay we developed in this study, we tested 78 samples from the lesions of 34 AD patients (head and neck, trunk, and limbs). *Malassezia* colonized the head and neck region most heavily, with 12.4 times more colonies than on the trunk (*P*=0.040), and 6.8 times more than on the limbs (*P*=0.002) (Fig. 2). *M. globosa* and *M. restricta* accounted for 30.3 to 34.7% and 45.4 to 51.0% of all *Malassezia* species, respectively. *M. globosa* and *M. restricta* colonized all of the AD patients, and also accounted for approximately 80% of all *Malassezia* colonization. These results suggest that they play a major role in exacerbating AD. Indeed, the levels of specific IgE antibody

Table 1. Oligonucleotide sequences of primers and probes used in this study

| Primer and probe              | Sequence (5' to 3')           | Amplicon size (bp) |
|-------------------------------|-------------------------------|--------------------|
| All <i>Malassezia</i> species |                               | 72                 |
| Mala-F (forward primer)       | CTAAATATCGGGGAGAGACCGA        |                    |
| Mala-R (reverse primer)       | GTACTTTTAACTCTCTTTCCAAAGTGCTT |                    |
| Mala-MGB (probe)              | FAM-TTCATCTTTCCCTCACGGTAC-MGB |                    |
| <i>M. globosa</i>             |                               | 75                 |
| M.glob-F (forward primer)     | GGCCAAGCGCGCTCT               |                    |
| M.glob-R (reverse primer)     | CCACAACCAAATGCTCTCCTACAG      |                    |
| M.glob-MGB (probe)            | FAM-ATCATCAGGCATAGCATG-MGB    |                    |
| <i>M. restricta</i>           |                               | 78                 |
| M.rest-F (forward primer)     | GGCGGCCAAGCAGTGTTT            |                    |
| M.rest-R (reverse primer)     | AACCAAACATTCTCTTTAGGTGA       |                    |
| M. rest-MGB (probe)           | FAM-TTCTCCTGGCATGGCAT-MGB     |                    |

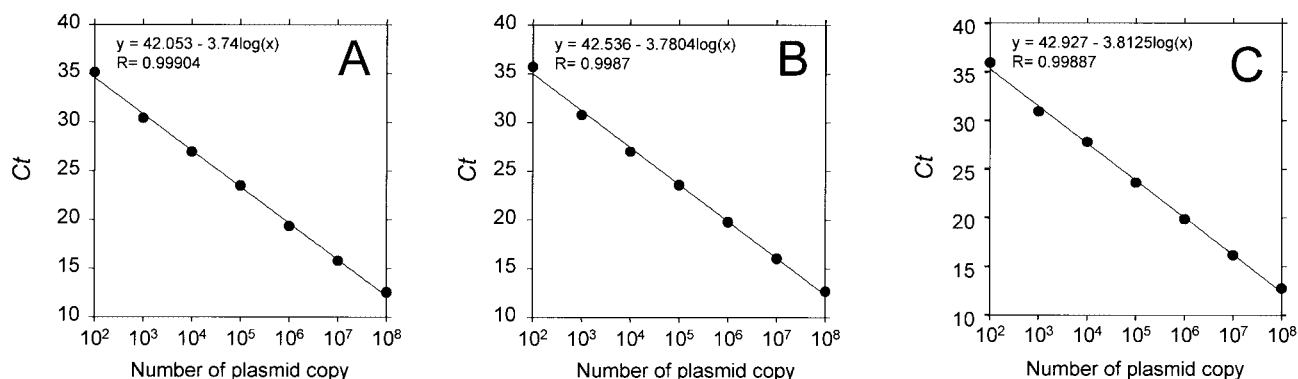


Fig. 1. Standard curves generated from known numbers of plasmid ( $10^2$ – $10^8$  copies). A, *Malassezia*-specific primers and probe sets. This line was plotted using a *M. globosa* plasmid as representative; B, *M. globosa*-specific primers and probe sets; C, *M. restricta*-specific primers and probe sets.

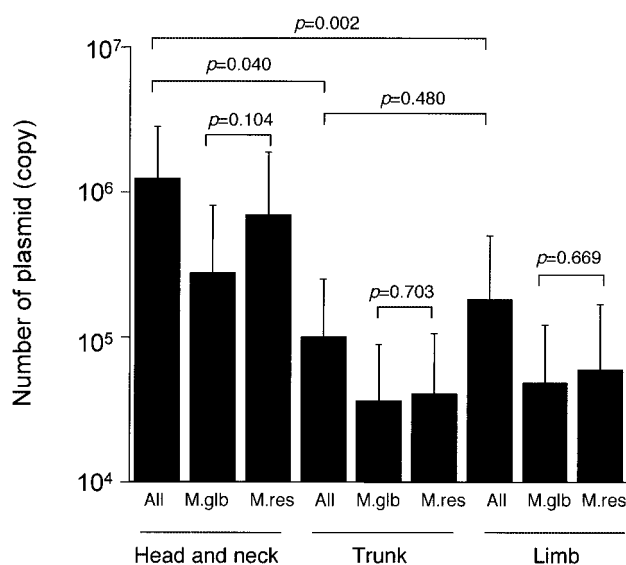


Fig. 2. Distribution of *Malassezia* by body site of skin of patients with atopic dermatitis. All, all *Malassezia* species; M. glb, *Malassezia globosa*; M. res, *Malassezia restricta*.  $P < 0.05$  is statistically significant (*t*-test).

against these two species are higher than those of specific IgE antibody against other *Malassezia* species (unpublished data). Previously, we demonstrated that *M. globosa* and *M. restricta* were the major cutaneous flora in AD patients qualitatively, and the present study showed that they were the major flora quantitatively using a real-time PCR assay. Since isolation media and procedures do not influence our assay, the real-time PCR assay is a reliable, appropriate, culture-independent quantitative method. Antifungal treatment of AD has been investigated because *Malassezia* is one of the exacerbating factors of AD. In addition to evaluating clinical improvement, our assay method enables the evaluation of the rate of decolonization. *M. globosa* and

*M. restricta* are also the major cutaneous flora in other *Malassezia*-related dermatitis: seborrheic dermatitis and pityriasis versicolor (6, 10). The quantitative analysis of cutaneous *Malassezia* in patients with these dermatitides is also of interest.

To our knowledge, this is the first report of the use of real-time PCR as an alternative to fungal culture for quantifying of the distribution of cutaneous *Malassezia* species.

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