

Molecular Analysis of *Malassezia* Microflora on the Skin of Atopic Dermatitis Patients and Healthy Subjects

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Members of the genus *Malassezia*, lipophilic yeasts, are considered to be one of the exacerbating factors in atopic dermatitis (AD). We examined variation in cutaneous colonization by *Malassezia* species in AD patients and compared it with variation in healthy subjects. Samples were collected by applying transparent dressings to the skin lesions of AD patients. DNA was extracted directly from the dressings and amplified in a specific nested PCR assay. *Malassezia*-specific DNA was detected in all samples obtained from 32 AD patients. In particular, *Malassezia globosa* and *M. restricta* were detected in approximately 90% of the AD patients and *M. furfur* and *M. sympodialis* were detected in approximately 40% of the cases. The detection rate was not dependent on the type of skin lesion. In healthy subjects, *Malassezia* DNA was detected in 78% of the samples, among which *M. globosa*, *M. restricta*, and *M. sympodialis* were detected at frequencies ranging from 44 to 61%, with *M. furfur* at 11%. The diversity of *Malassezia* species found in AD patients was greater (2.7 species detected in each individual) than that found in healthy subjects (1.8 species per individual). Our results suggest that *M. furfur*, *M. globosa*, *M. restricta*, and *M. sympodialis* are common inhabitants of the skin of both AD patients and healthy subjects, while the skin microflora of AD patients shows more diversity than that of healthy subjects. To our knowledge, this is the first report of the use of a nested PCR as an alternative to fungal culture for analysis of the distribution of cutaneous *Malassezia* spp.

Members of the genus *Malassezia*, lipophilic yeasts, colonize the skin of the head, neck, and shoulders of humans and are one of the causative factors in pityriasis versicolor and seborrheic dermatitis (3). *Malassezia* species are also considered to be one of the factors that exacerbate atopic dermatitis (AD), based on the finding that AD patients (but not healthy subjects) have specific serum immunoglobulin E (IgE) antibodies against *Malassezia* spp. (9, 22, 23). Application of topical antimycotic agents to AD patients decreases *Malassezia* colonization and the severity of eczematous lesions (2), suggesting that *Malassezia* species play a role in AD. In addition, several candidate *Malassezia* antigens have been implicated in the pathogenesis of AD (10, 11, 16, 17, 19, 24).

The taxonomy of the genus *Malassezia* was recently revised, primarily by using rRNA gene sequences, into seven species: *M. furfur*, *M. globosa*, *M. obtusa*, *M. restricta*, *M. pachydermatis*, *M. slooffiae*, and *M. sympodialis* (4, 5, 6). *M. globosa*, *M. obtusa*, *M. restricta*, and *M. slooffiae* were formerly designated *M. furfur*. The frequency of isolation of each species and its correlation with the clinical manifestations of AD have not been well investigated. Studies examining colonization by *Malassezia* spp. may aid in the understanding of the mechanism of AD and the development of an effective treatment. Due to the difficulties inherent in culturing *Malassezia* spp., we analyzed the cutaneous *Malassezia* microflora directly from the skin lesions of AD patients by using a nested PCR.

MATERIALS AND METHODS

Subjects. Thirty-two AD outpatients at Juntendo University Hospital were included in this study. As a comparison group of healthy subjects, 18 students at Meiji Pharmaceutical University who were negative for anti-*Malassezia*-specific IgE antibody were also included.

Sample collection. *Malassezia* samples were collected by applying OpSite transparent dressings (3 by 7 cm; Smith and Nephew Medical Ltd., Hull, United Kingdom) to the skin of AD patients and healthy subjects. Samples were collected from skin lesions (erosive, erythematous, and lichenoid) on the scalps, backs, and napes of AD patients. Patients had been treated intermittently by topical application of medium- to high-strength steroid ointment in a petrolatum base. Samples were collected from the scalps and napes of healthy subjects.

DNA extraction. The collected OpSite dressing was placed in 1.5 ml of lysing solution (100 mM Tris-HCl [pH 8.0], 30 mM EDTA [pH 8.0], 0.5% sodium dodecyl sulfate) and incubated for 15 min at 100°C. The OpSite dressing was then removed from the tube, and the suspension 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-propanol, using Ethaminate (Nippon Gene, Toyama, Japan) as a precipitation activator. The DNA pellet was resuspended in 50 µl of TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]). An unused OpSite dressing was used as a negative control.

Detection of *Malassezia* DNA by nested PCR. Nested PCR was conducted by using two sets of primers as shown in Table 1. The species-specific primers were derived from the internal transcribed spacer region of the rRNA gene (13). Internal transcribed spacer sequences were obtained from GenBank (accession numbers AB019329 to AB019350). Extracted DNA (20 µl) from each sample was added to 30 µl of the PCR master mixture, which consisted of 5 µl of 10× PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM MgCl₂; Takara Inc., Shiga, Japan), 4 µl of 200 µM deoxynucleoside triphosphates (an equimolar mixture of dATP, dCTP, dGTP, and dTTP; Takara), 30 pmol of each primer, and 2.5 U of Ex Taq DNA polymerase (Takara). PCR was performed in a thermocycler (model 9700; Applied Biosystems, Foster City, Calif.) with an initial denaturation of 94°C for 3 min, followed by 30 cycles of 30 s at 94°C, 1 min at 57°C, and 50 s at 72°C and a final extension at 72°C for 10 min. In the nested PCR step, 1 µl of the first amplification product was added to a new reaction mixture with the same composition as the first. The PCR procedure consisted of an initial denaturation of 94°C for 3 min, followed by 30 cycles of 30 s at 94°C, 1 min at 62°C, and 40 s at 72°C and a final extension at 72°C for 10 min. The PCR

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products were cloned by using a TA Cloning Kit (Invitrogen Corp., Carlsbad, Calif.) and sequenced with an ABI PRISM Cycle Sequencing Kit (Applied Biosystems) in accordance with the manufacturer's instructions.

Specificity of the PCR assay. Table 2 shows the strains used to investigate the specificity of the PCR assay. For basidiomycetous yeasts, DNA was extracted by the method of Makimura et al. (12). For ascomycetous yeasts, a DNA extraction kit (Nucleon MiY; Amersham International plc, Little Chalfont, United Kingdom) was used.

Anti-*Malassezia* IgE antibody detection. Specific IgE antibodies in the sera of AD patients and healthy controls were assayed by using AlaSTAT (Nippon DPC, Tokyo, Japan). Reactivity was defined in terms of antibody titers (14) as positive (more than 0.35 IU/ml) or negative (less than 0.35 IU/ml).

Statistical analysis. Fisher's exact probability test was applied to analyze the differences in *Malassezia* DNA detection between AD patients and healthy subjects.

RESULTS

Sensitivity and specificity of the nested PCR assay. The sensitivity of the PCR assay using two pairs of nested oligonucleotides was examined by using purified *Malassezia* DNAs from cultures. The limit of detection of purified DNA by the nested PCR assay was 1 fg. The species specificity of the nested PCR assay is shown in Table 2. Primers for the differentiation *M. furfur* from *M. obtusa* could not be prepared, since the *M. furfur* primer also amplified *M. obtusa* DNA. Therefore, all of the PCR products amplified by the primers for *M. furfur* and *M. obtusa* were cloned and the DNA was sequenced. The primers for the other four *Malassezia* species were species specific and amplified DNA only from the target *Malassezia* species. The primers did not amplify the DNA of other clinically relevant pathogenic yeast species or from *Staphylococcus aureus*, a common skin-colonizing bacterium.

Detection of *Malassezia* DNA. Table 3 shows the rates of *Malassezia* DNA detection in samples from AD patients and healthy subjects. We collected 58 samples from skin lesions (erosive, erythematous, and lichenoid) of 32 AD patients. DNAs of *M. restricta* and *M. globosa* were detected in 61.5 to 86.7% and 73.3 to 80.8%, respectively, of the samples taken from the three different types of skin lesion, with DNAs of *M. sympodialis* and *M. furfur* detected less frequently (26.9 to 35.3% and 23.1 to 47.1%, respectively). *M. slooffiae* DNA was found in less than 6.7% of the samples, and neither *M. obtusa* nor *M. pachydermatis* DNA was detected in any of the 58 samples from AD patients. The extent of *Malassezia* flora appeared to be independent of the type of skin lesion in which it was detected. Overall, *Malassezia* DNA was present in 86.7 to 96.2% of the samples from each type of skin eruption. *M. restricta* and *M. globosa* were the most common species detected in AD patients (87.5 and 93.8%, respectively), followed by *M. sympodialis* and *M. furfur* (40.6%). In healthy subjects, the DNAs of *M. restricta*, *M. globosa*, and *M. sympodialis* were detected in 61.1, 44.4, and 50.0%, respectively, and that of *M. furfur* was detected in 11.1%, while *M. slooffiae*, *M. obtusa*, and *M. pachydermatis* DNAs were not detected. Altogether, *Malassezia* DNA was detected in 77.8% of the samples from healthy subjects. A comparison of the DNA detection frequencies revealed that *Malassezia* DNA sequences, especially those of *M. restricta*, *M. globosa*, and *M. furfur*, were present at a significantly higher frequency in samples from AD patients than in those from healthy subjects ($P < 0.05$). In contrast, *M. sympodialis* was detected at the same rate in both AD patients and healthy subjects ($P > 0.05$).

TABLE 1. Oligonucleotides used in the nested PCR

Test, species, and primers used	Sequence (5'→3')	DNA fragment size (bp)
1st PCR for all 7 <i>Malassezia</i> species		
ITS1F-N	GGATCATTAGTGATTGCCTTTATA	
ITS4-R	TCCTCCGCTTATTGATATG	
2nd PCR for each <i>Malassezia</i> species		
<i>M. furfur</i>		230
M.f-F	CTACTCGCGTACAACGTCTCTG	
5.SS-R	TTCGCTGCGTTCTTCATCGA	
<i>M. globosa</i>		270
M.gl-F	CAATAAGTGTGTCTCTGCGG	
5.SS-R	TTCGCTGCGTTCTTCATCGA	
<i>M. obtusa</i>		180
M.ob-F	ACCCGTGTGCACACTGTTGAG	
5.SS-R	TTCGCTGCGTTCTTCATCGA	
<i>M. pachydermatis</i>		220
M.pa-F	CTGCCATACGGATGCGCAAG	
5.SS-R	TTCGCTGCGTTCTTCATCGA	
<i>M. restricta</i>		320
M.rt-F	CTTGGTTGGACCGTCACTGG	
M.rt-R	AGGCGGATGCAAAGTGCTC	
<i>M. slooffiae</i>		230
M.sl-F	ACGCACGCTAACACAACGTG	
5.SS-R	TTCGCTGCGTTCTTCATCGA	
<i>M. sympodialis</i>		190
M.sy-F	CGGACGCAAACACGTCTCTG	
5.SS-R	TTCGCTGCGTTCTTCATCGA	

Diversity of *Malassezia* species among individuals. The number of *Malassezia* species detected in each individual is shown in Fig. 1. Samples from AD patients contained, on average, 2.7 ± 0.9 species, compared with an average of 1.8 ± 1.0 species in samples from healthy subjects. Additionally, AD patients showed increased sensitization to *Malassezia* antigens (Fig. 2). Anti-*Malassezia* IgE antibody was detected (>0.35 IU/ml) in 29 (91%) of the 32 AD patients, while none of the healthy subjects showed a positive reaction ($P < 0.001$). This corroborates previous observations of specific IgE antibody in AD patients and healthy controls (7, 14, 18, 20, 23).

DISCUSSION

Since the *Malassezia* taxonomy was revised, only a few studies have examined human *Malassezia* microflora, although *M. furfur* has long been thought to be a major component of the microflora of human skin. Leeming et al. (8) analyzed the occurrence of *Malassezia* species in 20 patients with pityriasis versicolor. They reported that *Malassezia* species were isolated from the skin surfaces of the foreheads (95%) and backs (100%) of these patients. Three species were detected, *M. restricta*, *M. globosa*, and *M. sympodialis*. The predominant species colonizing each site varied; e.g., *M. restricta* (95%) was isolated mainly from the forehead whereas *M. sympodialis* (95%) was predominant in samples taken from other surface areas of the head. Aspiroz et al. (1) isolated 120 *Malassezia* strains from the chest, back, and scalp areas of 38 healthy

TABLE 2. Specificity of primers for seven *Malassezia* species

Species and strain(s):	Specificity of oligonucleotide primers ^b for:						
	<i>M. furfur</i>	<i>M. globosa</i>	<i>M. obtusa</i>	<i>M. pachydermatis</i>	<i>M. restricta</i>	<i>M. slooffiae</i>	<i>M. sympodialis</i>
<i>Malassezia furfur</i>							
CBS 1878 ^a	+	—	+	—	—	—	—
CBS 7981	+	—	+	—	—	—	—
Isolate MF1	+	—	+	—	—	—	—
Isolate MF2	+	—	+	—	—	—	—
Isolate MF3	+	—	+	—	—	—	—
<i>Malassezia globosa</i>							
CBS 7966	—	+	—	—	—	—	—
CBS 7990	—	+	—	—	—	—	—
Isolate MG1	—	+	—	—	—	—	—
Isolate MG2	—	+	—	—	—	—	—
Isolate MG3	—	+	—	—	—	—	—
<i>Malassezia obtusa</i>							
CBS7876	+	—	+	—	—	—	—
<i>Malassezia pachydermatis</i>							
CBS 1879	—	—	—	+	—	—	—
CBS 7044	—	—	—	+	—	—	—
<i>Malassezia restricta</i>							
CBS 7991	—	—	—	—	+	—	—
CBS 7877	—	—	—	—	+	—	—
Isolate MR1	—	—	—	—	+	—	—
Isolate MR2	—	—	—	—	+	—	—
Isolate MR3	—	—	—	—	+	—	—
<i>Malassezia slooffiae</i>							
CBS 7956	—	—	—	—	—	+	—
CBS 7972	—	—	—	—	—	+	—
<i>Malassezia sympodialis</i>							
CBS 7222	—	—	—	—	—	—	+
CBS 7977	—	—	—	—	—	—	+
Isolate MS1	—	—	—	—	—	—	+
Isolate MS2	—	—	—	—	—	—	+
Isolate MS3	—	—	—	—	—	—	+
<i>Candida albicans</i> CBS 562	—	—	—	—	—	—	—
<i>Candida glabrata</i> CBS 160	—	—	—	—	—	—	—
<i>Candida guilliermondii</i> CBS 566	—	—	—	—	—	—	—
<i>Candida parapsilosis</i> CBS 604	—	—	—	—	—	—	—
<i>Cryptococcus albidus</i> CBS 142	—	—	—	—	—	—	—
<i>Rhodotorula mucilaginosa</i> CBS 17	—	—	—	—	—	—	—
<i>Trichosporon asahii</i> CBS 2479	—	—	—	—	—	—	—
<i>Staphylococcus aureus</i>							
Isolate SA1	—	—	—	—	—	—	—
Isolate SA2	—	—	—	—	—	—	—

^a Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.^b +, product obtained; —, no product obtained.TABLE 3. Detection of *Malassezia* DNA in AD patients and healthy subjects

Subjects	No. of patients	No. of samples	Detection of DNA (%) of:							
			<i>Malassezia</i> spp.	<i>M. restricta</i>	<i>M. globosa</i>	<i>M. sympodialis</i>	<i>M. furfur</i>	<i>M. slooffiae</i>	<i>M. obtusa</i>	<i>M. pachydermatis</i>
AD patients	32		100 ^a	87.5	93.8	40.6	40.6	6.3	0	0
Types of lesions:										
Erosive	15	17	88.2 ^b	82.4	70.6	35.3	47.1	5.9	0	0
Erythematous	24	26	96.2 ^b	61.5	80.8	26.9	23.1	0	0	0
Lichenoid	13	15	86.7 ^b	86.7	73.3	33.3	26.7	6.7	0	0
Total		58	91.4 ^b	74.1	75.9	31.0	31.0	3.4	0	0
Healthy persons	18	27	77.8 ^a	61.1	44.4	50.0	11.1	0	0	0
			81.5 ^b	59.3	48.1	40.7	3.7	0	0	0

^a Value is a percentage of the number of patients.^b Value is a percentage of the number of samples.

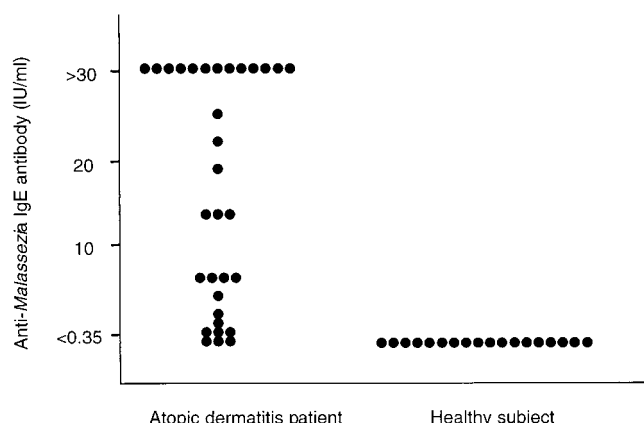


FIG. 2. Anti-*Malassezia* IgE antibody in 32 AD patients and in 18 healthy controls.

subjects. These strains were divided into three main *Malassezia* species, namely, *M. restricta*, *M. globosa*, and *M. sympodialis*. These two studies suggest that patients with pityriasis versicolor and healthy subjects have three *Malassezia* species on their skin in common. Our results confirmed that these three species predominate on the skin of healthy subjects. Although neither Leeming et al. (8) nor Aspiroz et al. (1) described the isolation of strains of *M. furfur* from human skin, we commonly found this species on the skin of both AD patients and healthy subjects. Recently, Nakabayashi et al. (15) compared *Malassezia* species by using colonies collected from the skin of patients with AD, seborrheic dermatitis, or pityriasis versicolor and normal subjects. In 13 (46%) of the 28 AD patients, *Malassezia* species were isolated from skin lesions, including *M. furfur* (21%), *M. globosa* (14%), *M. sympodialis* (7%), and *M. slooffiae* (4%). *M. restricta* was not isolated from the AD patients but was isolated from 0 to 2% of the patients with seborrheic dermatitis, 0% of the patients with pityriasis versicolor, and 1% of the normal subjects. These results are very different from ours. The cause of the discrepancy is most likely the fact that isolation of a pure, single colony of a *Malassezia* species uncontaminated by other yeasts is sometimes difficult. In addition, the efficiency of culturing of *Malassezia* strains may de-

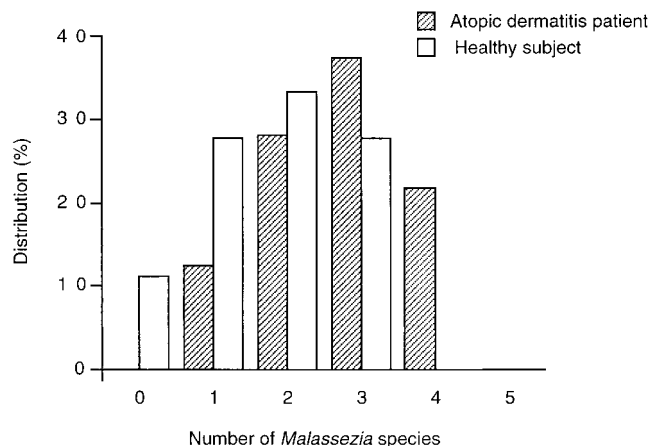


FIG. 1. The number of *Malassezia* species detected in AD patients and healthy subjects.

pend on the isolation medium employed. Indeed, Aspiroz et al. (1) pointed out that *Malassezia* recovery differed, depending on whether Dixon's or Leeming and Notman agar medium was used. Considering these facts, a molecular analysis-based non-culture method appears to be the most reliable and appropriate method by which to analyze in situ *Malassezia* distribution, since the isolation media and procedures used do not influence such a method.

In conclusion, we have determined that although *Malassezia* species are common on the skin of both AD patients and healthy subjects, the production of antibodies differs significantly between AD patients and healthy subjects. We feel, as do Terui et al. (21), that this is due to the disrupted barrier function of the skin surface and the effects of scratching-induced sensitization to the organisms.

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