

Cutaneous *Malassezia* flora in atopic dermatitis differs between adults and children

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Summary

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Conflicts of interest

None declared.

Background *Malassezia* species are suspected to be involved in the development of skin lesions in atopic dermatitis (AD) when the response of adult AD to anti-inflammatory treatments is poor. However, a comparative analysis of *Malassezia* flora between adults and children with AD has not been performed.

Objectives To compare the cutaneous *Malassezia* flora between adults and children with AD.

Methods Scale samples were collected from skin lesions of 58 patients with AD in the head and neck regions (28 males and 30 females; 31 children and 27 adults), and fungal DNA was extracted from the samples directly. The number and identities of the *Malassezia* species were analysed with high accuracy using a polymerase chain reaction-based culture-independent method. The *in vivo* level of anti-*Malassezia* IgE antibody was also assayed.

Results *Malassezia restricta* was the predominant species in the children with AD, while both *M. restricta* and *M. globosa* predominated in the adults. The adults showed increased sensitization in terms of anti-*Malassezia*-specific IgE responses in the sera to both *M. globosa* and *M. restricta* in comparison with the children.

Conclusions The cutaneous *Malassezia* flora differs significantly between the two age groups.

Atopic dermatitis (AD) is a chronic, fluctuating, inflammatory skin disease that rarely presents in adulthood; it commonly occurs within the first 2 years of life. The worldwide prevalence of AD is 10–20% in children and 1–3% in adults.¹

Atopic dermatitis is a multifactorial disease in which both hereditary and environmental factors play a role. For example, AD patients with inherited disruptions in the skin barrier function can experience induction of IgE production and activation of Th2 cells and an exacerbation of the symptoms of AD after exposure to various environmental factors.^{2–7} Therefore, *Malassezia* species in patients with AD with these features serve not only as normal skin flora but also as an exacerbating factor. Recently, the number of patients with AD, particularly adults, who respond poorly to anti-inflammatory treatment has been increasing.⁸ The treatment with antifungal agents of patients who are affected mainly in the head and neck regions has been found to decrease *Malassezia* colonization and the severity of the AD lesions, which suggests that *Malassezia* species play a significant role in AD.^{9–11}

Currently, 11 species are recognized in the genus *Malassezia*. Several studies have examined which species are

involved in exacerbating AD.^{8,12,13} However, the results of culture-based analyses of cutaneous *Malassezia* flora in patients with AD have differed among studies. These discrepancies are likely to have occurred due to different isolation techniques and media and the varying growth characteristics of each species. Consequently, Sugita et al.^{14,15} have recently developed a molecular-based culture-independent method for analysing cutaneous *Malassezia*. This method has enabled highly accurate analyses. *Malassezia* species are also associated with conditions other than AD, such as seborrhoeic dermatitis, pityriasis versicolor and psoriasis. The major species (*M. globosa* and *M. restricta*) are common, regardless of the skin disease,^{15–18} although the relative quantities of the species differ with different diseases.^{14,19} *Malassezia* have been found to colonize the head and neck regions to a greater extent than the trunk and limbs.¹⁴

The effects of ageing on the cutaneous *Malassezia* flora have not been accurately investigated. In the present study, we compared the *Malassezia* flora of two different age groups of patients with AD using a polymerase chain reaction (PCR)-based culture-independent method.

Materials and methods

Patients and sample collection

Fifty-eight individuals from two different age groups were sampled in this study. The two groups consisted of children under 15 years of age (mean $\pm$ SD 9.7 ± 1.5 years; 16 boys and 15 girls) and adults over 16 years of age (mean $\pm$ SD 36.1 ± 9.5 years; 12 men and 15 women). The patients were all outpatients at Juntendo University Nerima Hospital, Japan, and had been diagnosed with AD according to the criteria of Hanifin and Rajka.²⁰

Scale samples were collected from skin lesions by applying OpSiteTM transparent dressings (3×7 cm; Smith and Nephew Medical, Hull, U.K.) to the neck. As scale samples were obtained only from the neck, the severity of the AD was not defined with the SCORAD index, but instead was defined according to the surface of the involved skin, i.e. grade 1, scale only without inflammation; grade 2, slight erythema and scale; grade 3, erythema, scale, with a few papules and scratch marks; grade 4, erythema with swelling, oedema, infiltration and lichenification. Patients had not applied topical corticosteroids or antifungal agents for at least the preceding 4 weeks. The study was approved by the Institutional Review Board of Juntendo University Nerima Hospital, and written informed consent was obtained from each patient.

DNA extraction

The collected OpSite dressings were placed in lysing solution [100 mmol L^{-1} Tris-HCl pH 8.0, 30 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA) pH 8.0, 0.5% sodium dodecyl sulphate] and incubated for 15 min at 100°C . The suspension was extracted with phenol-chloroform-isoamyl alcohol ($25 : 24 : 1$, v/v/v) and then with chloroform-isoamyl alcohol ($24 : 1$, v/v), and the DNA was precipitated by the addition of ethanol and 3 mol L^{-1} sodium acetate, with Ethatinmate (Nippon Gene, Toyama, Japan) as a precipitation activator. The DNA pellet was resuspended in $30 \mu\text{L}$ of TE buffer (10 mmol L^{-1} Tris-HCl pH 8.0, 1 mmol L^{-1} EDTA pH 8.0) and stored at -20°C until use.

Detection of *Malassezia* species from scales

Eleven species are recognized in the genus *Malassezia*. Of these, *M. nana* and *M. pachydermatis* are specific to nonhuman animals and therefore were excluded from this study. The nine human-associated *Malassezia* species that colonized the scales were detected by nested PCR using the method of Morishita *et al.*¹⁷

Quantitative analysis of *Malassezia* in the scales

Among the *Malassezia* flora, two major cutaneous species, *M. restricta* and *M. globosa*, were further quantified using a universal or species-specific TaqMan probe according to the

method of Sugita *et al.*¹⁴ Amplification and detection were performed with an ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA, U.S.A.).

Detection of *Malassezia*-specific IgE antibodies

The levels of specific IgE antibodies against *M. globosa* and *M. restricta* were assessed using an enzyme-linked immunosorbent assay according to the method of Kato *et al.*²¹ Reactivity was defined in terms of antibody titres being positive ($\geq 0.35 \text{ IU mL}^{-1}$) or negative ($< 0.35 \text{ IU mL}^{-1}$).

Results

Cutaneous *Malassezia* flora in the different age groups

Both *M. globosa* and *M. restricta* were detected in all cases in the two age groups (Table 1). The other seven *Malassezia* species were detected across the ranges of 0–26% in the children and 0–40% in the adults. *Malassezia slooffiae* and *M. japonica* were not detected in either group. The cutaneous *Malassezia* flora of the adults was slightly more diverse than that of the children. Samples from the adults contained 3.2 ± 1.3 species (mean $\pm$ SD), compared with 2.8 ± 1.1 species in the samples from the children.

Quantitative analysis of *Malassezia* species in the two age groups

Malassezia colonization was evaluated based on plasmid copy number in a real-time PCR assay. Colonization with *Malassezia* species was 2.4-fold higher in the adults than in the children (mean $\pm$ SD $58\,827 \pm 58\,145$ vs. $24\,262 \pm 43\,196$ copies of plasmid DNA cm^{-2} ; $P < 0.05$) (Fig. 1a).

Malassezia globosa and *M. restricta* colonized all of the patients with AD and accounted for approximately 85.5% of the *Malassezia* isolates in the children and 70.6% in the adults (Fig. 1b). The ratios differed between the two groups: *M. restricta* colonization was 3.5-fold higher than *M. globosa* colonization in the

Table 1 Detection of *Malassezia* DNA in children and adults with atopic dermatitis

<i>Malassezia</i> species	Children (n = 23), n (%)	Adults (n = 20), n (%)
<i>M. globosa</i>	23 (100)	20 (100)
<i>M. restricta</i>	23 (100)	20 (100)
<i>M. dermatis</i>	3 (13)	7 (35)
<i>M. sympodialis</i>	2 (9)	4 (20)
<i>M. furfur</i>	6 (26)	2 (10)
<i>M. obtusa</i>	2 (9)	3 (15)
<i>M. yamatoensis</i>	6 (26)	8 (40)
<i>M. slooffiae</i>	0	0
<i>M. japonica</i>	0	0
Number of species detected (mean $\pm$ SD)	2.2 ± 1.1	3.2 ± 1.3

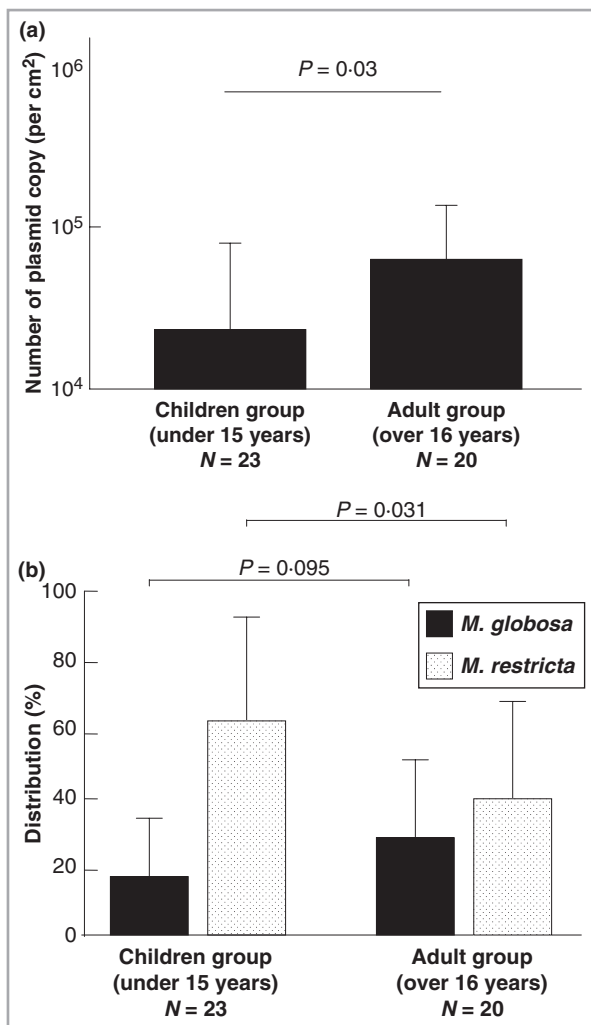


Fig 1. Distribution of *Malassezia* in lesional skin of adults and children with atopic dermatitis. (a) Amounts of all *Malassezia* species. The plasmid copy number cm⁻² is shown. (b) Distributions of the two major species, *M. restricta* and *M. globosa*.

children, whereas *M. restricta* colonization was 1.5-fold higher than that of *M. globosa* in the adults. Quantitative analysis showed that *M. restricta* was the dominant species in the children. There was no correlation between disease severity and the level of *Malassezia* colonization.

Anti-*Malassezia* IgE antibodies in the two age groups

In this experiment, 17 serum samples from adults and 20 serum samples from children were tested. Specific IgE antibodies against *M. globosa* and *M. restricta* were detected (> 0.35 IU mL⁻¹) in seven (41%) and seven (41%), respectively, of the 17 adults with AD, while eight (40%) and four (20%), respectively, of the 20 children with AD showed positive reactions. The levels of IgE antibodies against *M. globosa* and *M. restricta* in the adults (mean $\pm$ SD 8.8 ± 24.4 and 10.6 ± 25.9 IU mL⁻¹, respectively) were higher than those in the children (2.3 ± 6.5 and 0.6 ± 1.7 IU mL⁻¹, respectively;

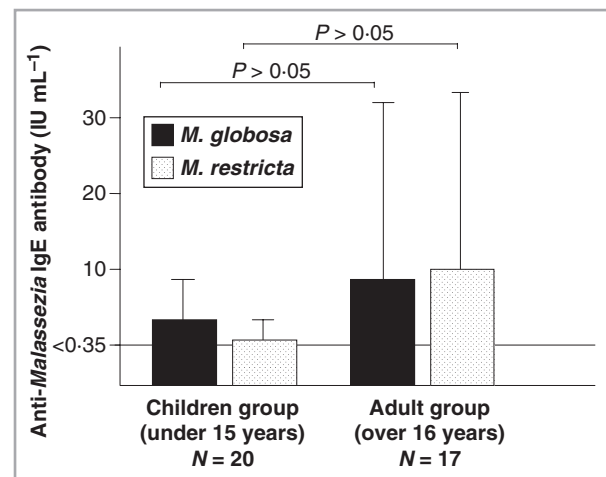


Fig 2. Levels of specific IgE antibodies against each *Malassezia* species in children and adults.

$P > 0.05$; Fig. 2). There was no correlation between the severity of disease and the level of IgE antibodies.

Discussion

The prevalence of AD has increased continuously over the last 30 years, especially in Western industrialized countries, where it occurs in up to 20–37% of the population.²² It has been suggested that this increasing prevalence results mainly from environmental factors, such as increased air pollution, indoor exposure to house dust mite antigens in less-ventilated modern homes, and dietary changes.⁸ The relationship between decreased exposure to microbial antigens associated with a Western lifestyle and the increasing severity and prevalence of AD has become known as the 'hygiene hypothesis'.²³ It has also been suggested that microorganisms play a role in AD pathogenesis.

Bacterial colonization with *Staphylococcus aureus* is the most common skin infection in AD ($> 90\%$ of patients compared with 5% of normal individuals) and occurs on both lesional and, to a lesser extent, nonlesional AD skin.^{24,25} Furthermore, a causal relationship appears to exist between the number of bacteria present on the skin and the severity of disease in patients with AD.

Recently, several researchers have investigated the cutaneous *Malassezia* microflora, using culture-based methods.^{26–28} However, the results have differed across studies. Sugita *et al.*^{14,15} have developed a PCR-based, culture-independent method to analyse the cutaneous *Malassezia* microflora with a high level of accuracy. Using this method, *M. restricta* and *M. globosa* have been found to be the most common species in adult patients with AD (87.5% and 93.8%, respectively), while the other species are detected in fewer than 40% of cases.¹⁵ The numbers of *M. restricta* and *M. globosa* on the skin of patients with AD are similar.¹⁴

In the present study, we quantified all the *Malassezia* species, including the two major species (*M. restricta* and *M. globosa*), in

AD scales of lesional skin using a highly accurate real-time PCR assay and compared the *Malassezia* flora between the two different age groups. *Malassezia* species were found to colonize adults to a greater extent than children, as shown in Figure 1(a). This may reflect the fact that, compared with adults, children have very little sebum on their skin.²⁹ Of the total *Malassezia* colonization, *M. restricta* was the dominant species in children, whereas the numbers of *M. restricta* and *M. globosa* were similar in the adults. Their relative compositions differed quantitatively between the two age groups, and this difference was probably due to different lipid classes or compositions.^{30,31}

The higher anti-*Malassezia* IgE antibody levels among adults compared with children with AD may reflect cumulative exposure.³² In the present study, we divided the patients into two groups, children (under 15 years of age) and adults (over 16 years of age); the latter group showed a higher sensitization to the antigens of both *M. globosa* and *M. restricta* than the former. However, when we divided the patients into children (under 12 years of age) and adults (over 13 years of age), specific IgE antibody against *M. restricta* was significantly associated with adults with AD ($P < 0.05$).

A Th2 cytokine profile predominates at birth. Microbial antigens induce immune deviations from a Th2 to a Th1 type profile, and adults maintain a Th1/Th2 balance.⁸ It has been suggested that exposure to microorganisms early in life is necessary for patients with AD to acquire immunocompetence. However, compared with children, this tends to become an exacerbating factor in adult patients with AD. *Malassezia* species were found to colonize the head and neck region most heavily.¹⁴ Given this observation, we suggest that antifungal agents may improve lesions in adult patients with AD who are affected mainly in the head and neck regions and who respond poorly to anti-inflammatory treatments.^{9–11}

In the present study, we evaluated the changes in *Malassezia* flora between different age groups of AD patients using a PCR-based, culture-independent method with high accuracy. Our results show that *M. restricta* is the predominant species in children with AD, whereas both *M. restricta* and *M. globosa* predominate in adults. However, further studies are needed to clarify the relationship between AD and *Malassezia* yeasts.

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