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Original article

IgE-reactivity to seven *Malassezia* species

Background: *Malassezia* yeasts play a role in the pathogenesis of atopic eczema/dermatitis syndrome (AEDS). The revised genus *Malassezia* includes several species which all are natural habitants of the human skin. In this study, we evaluated the presence of immunoglobulin E (IgE) antibodies to different *Malassezia* spp. in AEDS patients to allow optimization of the characterization of the IgE antibody profile of IgE-associated AEDS.

Methods: Ninety-six adult patients, with a clinical diagnosis of AEDS, were included in the study. Seventeen of the patients had IgE antibodies to *M. sympodialis*, ATCC 42132 (m70 ImmunoCAP, Pharmacia, Diagnostic AB, Uppsala, Sweden). The IgE antibodies to seven *Malassezia* spp. were measured and inhibition immunoblotting was performed to investigate whether *M. sympodialis* contains all the allergen components present in the other *Malassezia* spp.

Results: Twenty per cent of 79 AEDS patients with a negative m70 ImmunoCAP test had IgE antibodies to at least one of the other six *Malassezia* spp. tested. Our inhibition studies indicated that *Malassezia* spp. to a great extent, share allergenic determinants. However, *Malassezia* species also contained species-specific allergens.

Conclusion: The use of only one species of *Malassezia* is not sufficient to detect all patients IgE sensitized to *Malassezia*. To obtain an optimal allergen preparation both common allergenic components as well as species-specific allergens have to be considered.

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The genus *Malassezia* comprises yeasts that are natural habitants of the human skin and warm-blooded animals (1). In 1996 the genus was revised to include seven species, *M. sympodialis*, *M. globosa*, *M. restricta*, *M. slooffiae*, *M. furfur*, *M. obtusa*, and *M. pachydermatis* (2). The only non-lipid-dependent member of the genus is *M. pachydermatis*, while the remaining six species require long-chain fatty acids for *in vitro* growth (2, 3). *Malassezia* species (spp.) are considered to cause skin infections such as pityriasis versicolor and pityrosporum folliculitis (4–7) and they play an important role in the pathogenesis of seborrhoeic dermatitis and 'atopic dermatitis' (8–12 and reviewed in 13).

The European Academy of Allergology and Clinical Immunology (EAACI) has, based on the proposal of its Nomenclature Task Force, and with representation from the EAACI Dermatology Section, decided to recommend the term atopic eczema/dermatitis syndrome (AEDS) for 'atopic dermatitis' (14). Atopic eczema/dermatitis syndrome is a chronic inflammatory skin disease with a wide variety of clinical manifestations. Up to 65% of patients with a clinical diagnosis of AEDS may have immunoglobulin E (IgE) antibodies specific to *Malassezia* yeasts (8–10, 12, 13) and treatment with ketoconazole can improve the eczema and at the same time decrease the allergen specific IgE antibody and total serum IgE levels (15, 16).

Recently, *M. furfur*/*Pityrosporum orbiculare* strain no. 42132, American Type Culture Collection (ATCC) was classified by biochemical characterization as *M. sympodialis* (17, J. Faergemann, personal communication). This strain has been used in several studies (13) and is the allergen source for the production of the m70 ImmunoCAP by Pharmacia, Diagnostic AB (Uppsala, Sweden) for measuring IgE antibodies specific for *Malassezia* in serum. Extract obtained from *M. sympodialis* induces higher T-cell responses in AEDS patients than in healthy controls (18) and positive skin atopy patch-test reactions to *M. sympodialis* have been found in AEDS patients (19). The aim of this study was to evaluate the presence of IgE antibodies to different *Malassezia* spp. in AEDS patients to allow optimization of the diagnosis of IgE-associated subgroup of AEDS.

Material and methods

Yeast strains, culture conditions and extract preparation

In addition to the *M. sympodialis* strain no. 42132 (ATCC), the following cultures from the CBS (Centraalbureau voor Schimmelcultures, Delft, the Netherlands) were used: *M. sympodialis* 7222, *M. globosa* 7966, *M. restricta* 7877, *M. slooffiae* 7956, *M. furfur*

Table 1. Characterization of the AEDS patients

AEDS patients	No.	Age years median (range)	Gender F/M†	Phadiatop positive no.‡	Head and neck distribution no.	Past or present R and /or A no.§	Total serum IgE (kU/l) median (range)¶	Elevated serum IgE no.¶	m70-specific IgE (kU/l) median (range)*
m70 Negative*	79	23 (18–56)	57/22	48	29	45	81 (2–1314)	29	–
m70 Positive*	17	25 (18–39)	9/8	17	14	16	1238 (23–5700)	15	8.3 (0.6–56)

* m70 ImmunoCAP™, allergen source *M. sympodialis* ATCC 42132 (Pharmacia Diagnostics AB).

† Female/male.

‡ Serum IgE reactivity to 11 common aeroallergens (Pharmacia Diagnostics AB).

§ Rhinoconjunctivitis and/or asthma.

¶ Normal reference range: 1.3–263 kU/l for 5–20-year-old individuals and 1.6–122 kU/l for adults (Pharmacia Diagnostics AB).

7019, *M. obtusa* 7876, and *M. pachydermatis* 1879. All strains were cultured on modified Dixon agar (2) for 4 days at 32°C. The yeast extracts were prepared from 4-day-old cultures as previously described (20). Briefly, the cells were harvested and freeze-dried, re-suspended in phosphate-buffered saline (PBS), sonicated, centrifuged and sterile filtered (20). The protein concentration of the extracts was measured with bicinchoninic acid protein assay reagent (Pierce Chemical Company, IL) according to the manufacturer's instructions.

ImmunoCAP

Serum samples were analysed for total serum IgE levels, and IgE antibody to m70 (*M. sympodialis*, ATCC 42132), and a mix of 11 common aeroallergen sources (Phadiatop: Cladosporium, *Dermatophagoides farinae*, *D. pteronyssinus*, cat, dog, horse, birch, timothy grass, mugwort, olive, and *Parietaria judaica*) by the CAP System™ IgE FEIA and Phadiatop®, respectively (Pharmacia Diagnostics AB) (Table 1). Extracts of the other *Malassezia* spp. were covalently coupled to the cellulose solid phase (ImmunoCAP) (Pharmacia Diagnostics AB) and IgE antibody was measured in the Pharmacia CAP System according to the manufacturer's instructions. A positive ImmunoCAP result was defined as a value of at least 0.35 kU/l. No non-specific binding of IgE was found with a serum from a healthy individual with normal IgE concentration which spiked with myeloma IgE up to 1000 kU/l.

Patient sera

Seventeen m70 ImmunoCAP positive and 79 m70 ImmunoCAP negative serum samples from 96 adult patients, with a clinical diagnosis of AEDS (21), and mild, moderate, or severe dermatitis as scored on the scale proposed by Rajka and Langeland (22) were selected from a previous study (23). Recorded data including age, sex, head and neck distribution, presence of rhinoconjunctivitis or asthma along with the serological findings are given in Table 1. In addition, sera from five AEDS patients with positive m70 ImmunoCAP (median 20 kU/l, range 9.1–56 kU/l) and high total serum IgE levels (median 5500 kU/l, range 2300–8900 kU/l) were pooled for use in the inhibition analysis. The study was approved by the Ethics Committees at the Karolinska Hospital and Lund University Hospital.

Immunoblotting and inhibition assay

Equal amounts, based on protein content, of each of the *Malassezia* extracts (24 µg/mm gel) or Dixon broth as control were separated by electrophoresis on 7.5–20% polyacrylamide gels under reduced conditions. The gels were blotted onto polyvinylidene difluoride membranes (Millipore Corp., MA). The membranes were either

incubated with the serum pool (diluted 1 : 6 in PBS), or cut into strips and used for the inhibition assay. For immunoblotting inhibition *M. sympodialis*, ATCC 42132, was used to inhibit binding of IgE to allergens in the extracts of other *Malassezia* spp. The serum pool was incubated at room temperature for 2 h with different protein concentrations of *M. sympodialis* (3, 2, 1, 0.5, and 0.25 mg) or with 3 mg bovine serum albumin as a negative control. The mixtures were then incubated with the blotted membranes of various *Malassezia* extracts. Detection of IgE-binding components was performed as described previously (20). The intensity of the bands was recorded in a scanner and the percentage of the inhibition was analysed with the Image Analysis Systems Software, IASS, (Molecular Analyst Software, BIO RAD, CA).

Results

ImmunoCAP

Of the 79 AEDS patients negative to m70 (*M. sympodialis*, ATCC 42132), 16 (20%) were found to have IgE antibodies to one or more of the other *Malassezia* extracts. The *M. globosa* extract showed the most positive reactions (11/16, 69%) while the *M. slooffiae* extract was the least reactive (3/16, 19%) (Table 2). Among the 16 patients four were ImmunoCAP positive to only one extract (*M. globosa*, *M. restricta*, *M. obtusa*, or *M. pachydermatis*). Out of the 16 patients 12 (75%) had elevated total serum IgE, 14 (88%) had a positive Phadiatop and eight (50%) had head and neck dermatitis.

All the seventeen patients who were included because of a positive m70 ImmunoCAP had IgE antibodies to the *M. sympodialis* CBS 7222 extract (Table 2). Sixteen patients were ImmunoCAP positive to the other extracts except for *M. slooffiae* and *M. furfur* (Table 2). One patient with an IgE antibody level of 0.9 kU/l to m70 was ImmunoCAP positive only to the other *M. sympodialis* strain from CBS 7222 and not to the other species (Table 2), indicating that *M. globosa* and *M. restricta*, despite showing numerous IgE-binding components (Fig. 1), do not contain all the allergen components present in the *M. sympodialis* extract.

There was a significant correlation between total serum IgE and specific serum IgE to all seven *Malassezia* spp., using Spearman rank correlation ($r = 0.51–0.67$, $P < 0.001$). None of the spp. were more often correlated with head and neck manifestation.

Table 2. IgE antibodies to *Malassezia* spp. in sera from AEDS patients

Category*/class†	No. and (%) of patients	No. and (%) of patients positive to						
		<i>M. sympodialis</i> ‡	<i>M. globosa</i>	<i>M. restricta</i>	<i>M. slooffiae</i>	<i>M. furfur</i>	<i>M. obtusa</i>	<i>M. pachydermatis</i>
m70 pos/≥ 4	4 (24)	3 (18)	7 (41)	8 (47)	2 (12)	4 (24)	7 (41)	6 (35)
/3	7 (41)	7 (41)	6 (35)	5 (29)	7 (41)	7 (41)	6 (35)	5 (29)
/2	5 (29)	5 (29)	3 (18)	2 (12)	6 (35)	3 (18)	3 (18)	5 (29)
/1	1 (6)	2 (12)	0 (0)	1 (6)	0 (0)	0 (0)	0 (0)	0 (0)
Total pos:	17 (100)	17 (100)	16 (94)	16 (94)	15 (88)	14 (82)	16 (94)	16 (94)
m70 neg/3	–	0 (0)	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
/2	–	2 (3)	6 (8)	3 (4)	1 (1)	0 (0)	4 (5)	4 (5)
/1	–	3 (4)	4 (5)	3 (4)	2 (3)	4 (5)	2 (3)	4 (5)
Total neg:	79	5 (7)	11 (14)	6 (8)	3 (4)	4 (5)	6 (8)	8 (10)
Total:	96	22 (23)	27 (28)	22 (23)	18 (19)	18 (18)	22 (23)	24 (25)

* m70 ImmunoCAP™, allergen source *M. sympodialis* ATCC 42132 (Pharmacia Diagnostics AB).

† The cut-offs for each ImmunoCAP class ≥ 4, 3, 2, and 1 are ≥ 17.5, 3.5, 0.7 and 0.35 kU/l, respectively.

‡ *M. sympodialis* CBS 7222.

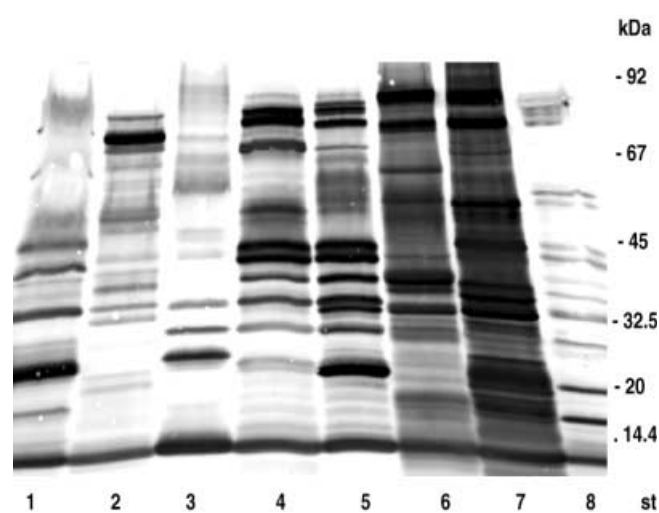


Figure 1. Immunoblotting analysis of *Malassezia* extracts. Binding of serum IgE (a pool of five sera, see M & M) to extracts of *M. obtusa* (lane 1), *M. pachydermatis* (lane 2), *M. furfur* (lane 3), *M. sympodialis* CBS 7222 (lane 4), *M. sympodialis* ATCC 42132 (lane 5), *M. globosa* (lane 6), *M. restricta* (lane 7), and *M. slooffiae* (lane 8) are shown. The molecular mass markers are indicated.

Immunoblotting and inhibition assay

Immunoblotting analysis indicated that the patterns of the bands in different *Malassezia* spp. show similarity with each other (Fig. 1), but it seems that *M. globosa* (lane 6) and *M. restricta* (lane 7) contain more IgE-binding components than the other *Malassezia* spp. The control Dixon medium did not show any band with the pooled serum (data not shown).

Extracts of *M. sympodialis* ATCC 42132 of different protein concentrations were used to inhibit the binding of IgE to *Malassezia* spp. allergens (Fig. 2A–H). When the inhibitor was used at the concentration of 0.5 mg,

66% of the IgE-binding to *M. sympodialis* CBS 7222 was inhibited (Fig. 2A, lane 4), and with 3 mg of inhibitor 79% inhibition was obtained (Fig. 2A, lane 7). The binding of serum IgE to *M. globosa*, *M. restricta*, *M. slooffiae*, *M. furfur*, *M. obtusa*, and *M. pachydermatis* was reduced by 22, 27, 37, 64, 82, and 53%, respectively, when the serum pool was preincubated with 0.5 mg of the inhibitor (Fig. 2B–G, lane 4). Increasing the concentration of the inhibitor to 3 mg resulted in inhibition of *M. globosa*, *M. restricta*, *M. slooffiae*, *M. furfur*, *M. obtusa*, and *M. pachydermatis* by 54, 60, 93, 87, 96, and 77%, respectively (Fig. 2B–G, lane 7). In the homologous inhibition, 82% extinction of the bands was obtained when 0.5 mg of *M. sympodialis* ATCC 42132 was used as inhibitor (Fig. 2H, lane 4), and this increased to 95% using 3 mg (Fig. 2H, lane 7). The results show that *M. sympodialis* ATCC 42132 at concentrations lower than 3 mg is a rather poor inhibitor of IgE binding to *M. globosa* and *M. restricta*, as the patterns were reduced by 60% or less even at the highest inhibitor concentration (Fig. 2B and C, lane 7). In contrast, the binding of IgE to *M. furfur*, *M. obtusa*, and *M. pachydermatis* was significantly reduced even with the lowest concentration of the *M. sympodialis* inhibitor (0.25 mg) (Fig. 2E–G, lane 3).

Discussion

As the genus *Malassezia* has been revised and expanded to include several species, all present on human skin, a need has arisen to consider the usage of correct species for the identification of IgE antibodies to *Malassezia* in patients with AEDS. In this study, we demonstrated that the use of only one species is not sufficient to detect all the patients with IgE antibodies to *Malassezia*. Twenty percent of 79 AEDS patients with a negative m70 ImmunoCAP test (*M. sympodialis* ATCC 42132) had IgE

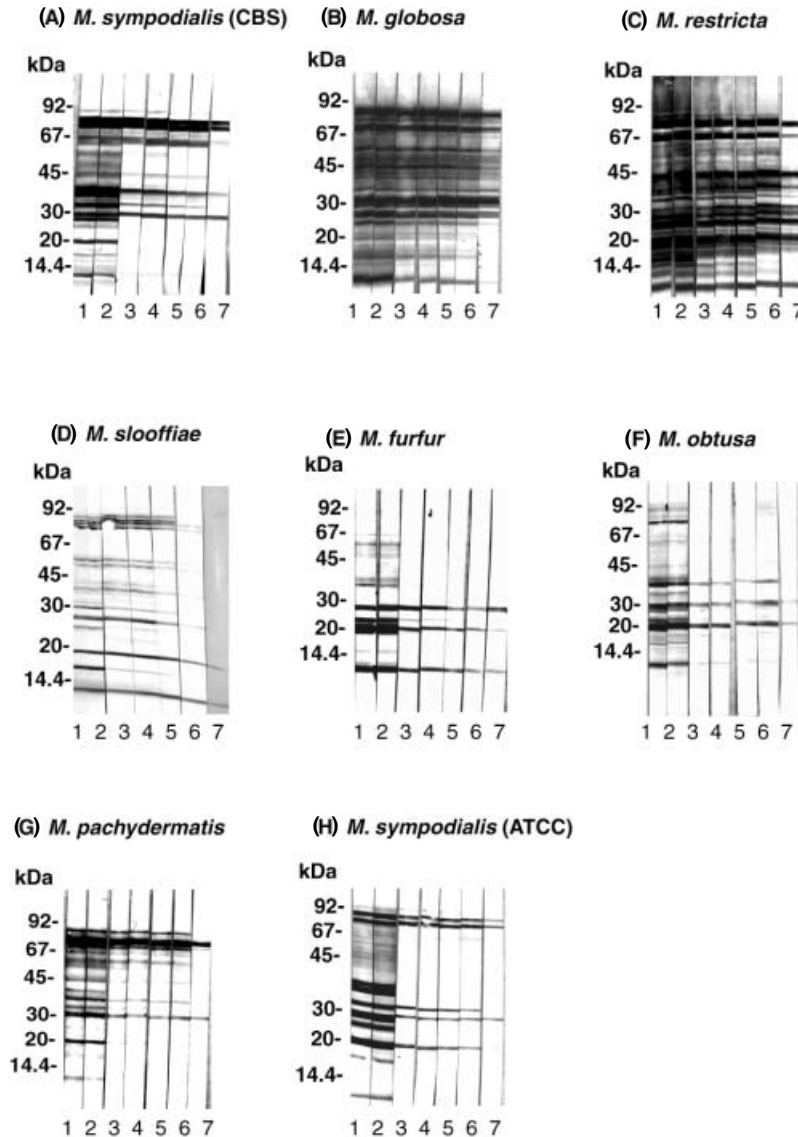


Figure 2. Inhibition of IgE binding to *Malassezia* spp. extracts by *M. sympodialis* ATCC 42132. A pool of five sera all with total serum IgE value ≥ 2300 kU/l and positive m70 ImmunoCAP = 9 kU/l were preincubated with 0.25, 0.5, 1, 2, and 3 mg protein (lane 3–7 respectively) of *M. sympodialis* ATCC 42132 extract or with 3 mg BSA (lane 2) as control. The preincubated sera were used to inhibit binding of IgE to the blotted membrane of different *Malassezia* spp. (A–H). The first lane in each set is the binding of the pool serum without inhibitor to different *Malassezia* spp. The molecular mass markers are indicated.

antibodies to other *Malassezia* spp. A number of studies have been published on the occurrence of *Malassezia* species on the skin of AEDS patients and normal controls. Among these, Sugita et al. used nested PCR and showed that *M. furfur*, *M. globosa*, *M. restricta*, and *M. sympodialis* are common inhabitants of the skin of both AEDS patients and healthy subjects (24). In that study, *M. globosa* and *M. restricta* were detected on the skin of 90% of the patients, *M. sympodialis* and *M. furfur* were detected on 40% of the patients, whereas *M. slooffiae* DNA was found in less than 7% of the patients and not in the healthy controls (24). Aruzuma-

nian showed *M. sympodialis* to be the most frequent species in both AEDS patients and healthy individuals and *M. globosa* was infrequently isolated from normal skin (25). However, isolation and detection of *Malassezia* spp. from the skin varies, based on several factors such as methods of detection, the efficiency of culturing of *Malassezia*, the use of different media, area of sampling, and geographical differences. Previous serological work by Koyama et al. on five different *Malassezia* spp. demonstrated that in patients with AEDS, 83% of sera had IgE antibodies to *M. globosa*, 74% to *M. sympodialis*, 65% to *M. furfur*, 56% to *M. restricta*, and 50% to

M. slooffiae (26). Although we have selected AEDS patients based on a previous m70 reactivity, our results show that IgE antibodies to all seven tested *Malassezia* spp. were present and most frequently to *M. globosa* (Table 2). This suggests that *M. globosa* should be also considered as one of the candidates for identification of IgE antibodies in AEDS patients suspected of *Malassezia* allergy. Several studies show that *M. slooffiae* is isolated less frequently from the skin (11, 24) which is in agreement with our finding that IgE antibodies to this species are less frequent.

Our inhibition studies indicate that *Malassezia* spp. to a great extent share allergenic determinants. We also observed that four patients had IgE antibodies to only one species, indicating the presence of species-specific allergenic epitopes. This is in concordance with Koyama's report that *Malassezia* species contained both species-specific and common allergenic components reacting with IgE antibodies (26).

Malassezia sympodialis, *M. globosa* and *M. restricta* have been reported to be more dominant on the skin of AEDS patients or healthy individuals (11, 24, 27–30). The IgE antibodies are also more frequently found with these species in AEDS patients (26). Our inhibition study shows that *M. sympodialis* does contain up to 60% of the IgE-binding components present in the *M. globosa* and *M. restricta* extracts. Therefore, we believe that *M. sympodialis* alone is not enough to detect IgE antibodies in patients with AEDS. A mixture of *M. sympodialis*, *M. globosa*, and *M. restricta* extracts could provide a broad spectrum of allergens for detection of IgE antibodies to *Malassezia*. Addition of recombinant species-specific allergens from other *Malassezia* spp. might even further broaden the screening for an IgE sensitization.

Five of our patients with negative m70 ImmunoCAP (*M. sympodialis* ATCC 42132) had IgE antibodies to *M. sympodialis* CBS 7222 (all with ImmunoCAP

value < 1.3 kU/l). This can be due to strain differences (one from CBS, the other from ATCC) or variations in extract preparation. Many efforts have been dedicated to cloning of the *Malassezia* allergens and production of pure recombinant allergens to overcome the problem with batch to batch variation (31–35, reviewed in 13). We have previously shown that individual recombinant allergens (Mal s 1, and Mal s 5–9) from *M. sympodialis* (previously denoted *M. furfur* ATCC 42132) will detect between 32 and 89% of the m70 ImmunoCAP positive patients (23). Addition of relevant individual recombinant allergens could thus improve the detection of serum IgE antibodies to *Malassezia* in patients with AEDS (23). It remains to be established to what extent the so far identified and cloned allergens from *M. sympodialis* ATCC 42132 are also present in the other *Malassezia* spp. Recently, Sugita et al. isolated a new member of the genus *Malassezia* from the skin of patients with AEDS and proposed the name *M. dermatis* for this novel species (36). Further investigations are required to clarify the presence of IgE reactivity to this species.

In conclusion our study shows that the use of only one species of *Malassezia* is not enough to detect all patients IgE sensitized to *Malassezia*. To obtain an optimal allergen preparation both common allergenic components as well as species-specific allergens have to be considered.

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