

Evaluation of *Candida albicans* allergens reactive with specific IgE in asthma and atopic eczema patients

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Summary

Candida albicans (*C. albicans*) produces important allergenic components which can induce allergic reactions in sensitised patients. The purpose of this study was to extract the *C. albicans* antigens for evaluating the specific anti-*Candida* IgE in sera of atopic eczema (AE) and asthmatic patients (AS). 95 AE, 85 AS, and 70 non-atopic cases were selected with sequential trials. *Candida albicans* antigens were prepared and then skin prick test (SPT), ELISA and IgE-immunoblotting tests were performed for all patients. Positive SPT reactions were obtained on 52.6% of AE and 54.1% of AS patients and 4.3% of healthy controls ($P < 0.05$). Using ELISA, specific anti-*C. albicans* IgE antibody was detected in 32.6% and 41.2% of patients with AE and AS, respectively. No specific IgE antibody was detected in healthy controls ($P < 0.05$). In SDS-PAGE, protein bands with molecular weights between 13 and 135 kDa were detected, and some of them reacted with specific IgE in immunoblotting. In AE patients, the most important allergenic components were 25, 34, and 57 kDa protein bands, whereas in AS, 22, 25, and 34 kDa protein bands were observed as major allergens. *Candida albicans* produces different allergenic components that can induce allergic reactions and may be pathogenetically important in patients with AE and AS.

Key words: *Candida albicans*, atopic eczema, asthma, skin prick test, IgE.

Introduction

Candida albicans, a commensal yeast, is the most common opportunistic fungal pathogen that causes a disseminated systemic infection in immunocompromised hosts.¹ In addition, *C. albicans* has been examined as a possible allergen in allergic diseases such as bronchial asthma^{2,3} and atopic eczema.⁴ Several studies of the antigenic components of *C. albicans* have been performed with allergic patients who are positive for IgE antibodies to *C. albicans*, and the characteristics of the IgE-binding components of *C. albicans* have been reported.^{5,6} Atopic eczema (AE) is a chronic, itching,

inflammatory skin disease, which is associated with asthma and/or hay fever.⁷ Atopic eczema is the most common chronic skin disease in children and adults.⁸ Genetic factors are important in the development of AE, but the exact hereditary pathway is still unknown.⁹ Infiltrates of activated Th-2 cells are found in the skin of patients with AE. An interesting observation in the skin of AE patients is the presence of a dendritic epidermal cell population that carries IgE receptors and specific IgE. Antigen presented by these cells may stimulate Th₂ cells to induce IgE production by B cells and hence contribute to the inflammatory changes in the skin.^{10,11} Previous studies have found positive skin prick test (SPT) and IgE-mediated hypersensitivity to *C. albicans* in 52%, 25–94%, 30% and 27% of patients with AE.^{5,12–14} Asthma (AS) is a multifactorial and complex disease in which allergic factors and non-allergic triggers interact and result in bronchial obstruction and inflammation.¹⁵ Allergic sensitisation is important in the development of asthma, and although links between inhalant allergy

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and AS have been known for many years, they have recently been re-emphasised.¹⁶ Moreover, there is a link between total IgE and asthma, which appears to be independent of allergen sensitisation.¹⁷ Shen *et al.* [6] and Savolainen *et al.* [4] identified at least 16 allergenic components of *C. albicans* with molecular weights ranging from 20 to 94 and from 16 to 135 kDa, respectively, reacting with IgE of serum samples from about 30 AS patients. Crude antigens of *C. albicans* have been used for the *in vitro* detection of IgE antibodies.¹⁸ Therefore, it would be useful to develop standard *C. albicans* antigens for IgE detection to define major antigenic components of this fungus. The goal of this study was to examine the antigenic components of *C. albicans* that reacted with IgE antibodies in patients with AS and AE.

Patients and methods

Strain and cultivation

Candida albicans (ATCC 10261) was used in this study. This isolate was grown in sabouraud glucose agar containing chloramphenicol at 30 °C for 3 days. The yeast suspension (10^5 cell ml^{-1}) was cultured in 200 ml of YPG broth (Yeast extract 1%, Peptone 2%, and Glucose 2%) and agitated in the shaker (150 rpm) at 37 °C for 48 h. At the end of incubation, yeast cells were harvested at 4 °C by centrifugation at 4500 *g* for 5 min and centrifuged three times with 0.15 mol l^{-1} PBS, pH 7.2, at 1400 *g* for 10 min and once with breaking buffer at 4000 *g* for 10 min, and then dialysed against 0.05 mol l^{-1} PBS (cut off = 12 kDa; Sigma, Poole, UK).^{10,19} In this study, all general chemical materials were purchased from Merck Co., Darmstadt, Germany.

Cell fractionation and crude extract preparation

This procedure was performed according to Kumar and Axelsen methods with a little modification.^{20,21} Yeast disruption was performed using two methods: (i) *Sonication*: A suspension of wet compressed yeast cells (55% w/v) was prepared in breaking buffer, pH 7.2, (62.5 mol l^{-1} Tris-HCl, 15% Glycerol, 1 mmol l^{-1} Dithioeritol and 0/2 mg ml^{-1} PMSF) and then subjected to cell disruption by sonication (UP 200 s; Dr Hielscher Sonicator Set, Deisenhofen, Germany) by keeping it in ice bath to prevent overheating. Cell disruption was carried out using 60% amplitude for 48 min (2 : 4 min pulse on : off basis) to achieve maximum disruption of cells. (ii) *Glass beads*: disruption was performed using glass beads (diameter, 1 mm) on a

vortex mixer for 1 min (1 : 5 min pulse on : off basis) until about 80–90% yeast cells were disrupted. After cell disruption, the crude extract was separated from intact cells and cell walls remaining by centrifugation at 6000 *g* for 15 min and then the supernatant was recentrifuged at 26 000 *g* for 15 min, subsequently ultracentrifuged at 105 000 *g* for 1 h. The clear supernatant obtained was dialysed overnight at 4 °C against distilled water and stored at –20 °C until used.

Determination of protein

Protein concentration of lyophilised sample was determined after reconstituting in 50 and 100 μl of distilled water by Bradford [22] method.

Patients and healthy controls

A total of 180 patients with AE (95) and AS (85) were enrolled in this study. The diagnosis of AE was based on the criteria of Hanifin and Rajka [7] and accurate assessment of the severity of AE was performed using SCORAD index according to Stalder *et al.* [23] and was determined by a dermatologist from the Department of Dermatology, Imam Khomeini Hospital, in which they were divided into three groups: 14 mild (<15), 42 moderate (15–40) and 39 severe (>40). Asthmatic patients were chosen as sequential trial, in the Tehran Allergy Clinic. Also, 70 healthy controls with no personal or familiar history of atopy were enrolled in the experiments. All subjects gave informed consent to participate in the study.

Skin prick test (SPT)

Skin prick tests were performed with 3 μl of *C. albicans* extract (protein concentration, 100 $\mu\text{g ml}^{-1}$) on the volar aspect of the forearm and pricked with a 27 gauge needle. A negative control solution containing PBS with 50% glycerin and a positive control solution containing 0.1% histamine were applied in the same way. The test was read after 15 min and any reaction with a mean weal diameter at least 2–3 mm greater than negative control was taken as positive.

ELISA test

The *C. albicans* extract was diluted in 0.05 mol l^{-1} carbonate-bicarbonate buffer, pH 9.5, and a suitable titre was chosen using checker board titration. One hundred microliter of the antigen (25 $\mu\text{g ml}^{-1}$) was added to the wells and stored overnight at 4 °C. The

wells were rinsed extensively with washing buffer (PBS-Tween 20, 0.05%), and then 150 µl of blocking buffer was added to the wells and microplate was stored at 37 °C for 2.5 h. The wells were rinsed again. Serum samples were diluted 1 : 4 in PBS-Tween 20-BSA 1% and 100 µl were added to each well and incubated with shaking at 37 °C for 2 h, followed by washing with PBS-Tween 20, 0.05%. Anti-human IgE conjugated with ALP (Sigma, St Louis, MO, USA) was diluted 1/2000 in PBS-Tween 20-BSA 1% and 100 µl was added to each well and incubated at 37 °C for 1.5 h and followed by washing with PBS-Tween 20-BSA 1%. Each well received 100 µl of a solution of 0.1 mol l⁻¹ diethanolamine (DEA), pH 9.8, 1 mmol l⁻¹ magnesium chloride and paranitrophenol phosphate (PNPD). After 30 min, the reaction was stopped with 50 µl of 3 mol l⁻¹ NaOH and the OD was read at 405 nm. Each sample was tested in duplicate. Sera of healthy individuals were used as negative control. OD405 values greater than the OD value of the mean obtained with healthy controls +3 standard deviations were considered positive.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis

The yeast extract was separated in a 17.5% separation gel with a 5% stacking gel in a discontinuous buffer system according to the method of Laemmli [24]. The extract was boiled for 5 min with a reducing sample buffer (containing 2-B-mercaptoethanol) and 10 µl of sample (containing 60 µg proteins) was loaded on a 0.5 cm gel. Molecular weight standard markers (Rainbow markers, 14.4 to 93 kDa; Amersham International PLC, Buckinghamshire, UK) were loaded on the gel. The gel was stained with commassie brilliant blue R-250 (Sigma).

IgE-Immunoblotting test

Separated proteins were transferred to nitrocellulose sheets (Schleicher and Shuell, Dassel, Germany) with a transphor apparatus (Hoefer, San Francisco, CA, USA) at 100 volts for 2.5 h with a transfer buffer (25 mmol l⁻¹ Tris, 192 mmol l⁻¹ Glycine, 0.03% SDS, and 20% Methanol, pH 8.3). The nitrocellulose papers were cut into 0.5 cm and incubated at 4 °C for overnight with TBS-Tween 20-BSA buffer (20 mmol l⁻¹ M, 0.15 mol l⁻¹ NaCl, 0.05% Tween 20, and 1.5% BSA, pH 7.5). The strips were washed three times with TBS-Tween 20 buffer at room temperature for 15 min. Sera were diluted 1 : 2 in TBS-Tween 20-BSA and incubated at room temperature for 16 h. After

the strips had been washed 3 times with TBS-Tween 20-BSA, anti-human IgE conjugated with ALP (Sigma) diluted 1 : 1000 in TBS-Tween 20-BSA was added to each strip. After a 5-h incubation, the strips were washed and followed by washing with alkaline phosphatase buffer (100 mmol l⁻¹ Tris-HCl, 100 mmol l⁻¹ NaCl, and 5 mmol l⁻¹ magnesium chloride) for 10 min. The immunoblot was considered positive if at least one protein band was visible.²⁵

Statistical analysis

Data were analysed by the chi-square test. A *P*-value of <0.05 was considered statistically significant.

Results

Skin prick test

Of 95 patients with AE and 85 AS patients, 50 (52.6%) and 46 (54.1%) patients reacted positively to *C. albicans* antigens, respectively, whereas there were only 3 (4.3%) positive reactions in healthy controls (Fig. 1). Significant difference was observed between patients (AE and AS patients) and healthy controls in SPT (*P* < 0.05). There were no significant differences in SPT results between patients (AE and AS patients) with respect to gender, age, familiar history, and AE or AS diseases. Positive SPT to *C. albicans* were mostly observed in patients aged between 15 and 24 years in the group of AE, and aged 35 and 44 years in the group of AS patients (Table 1).

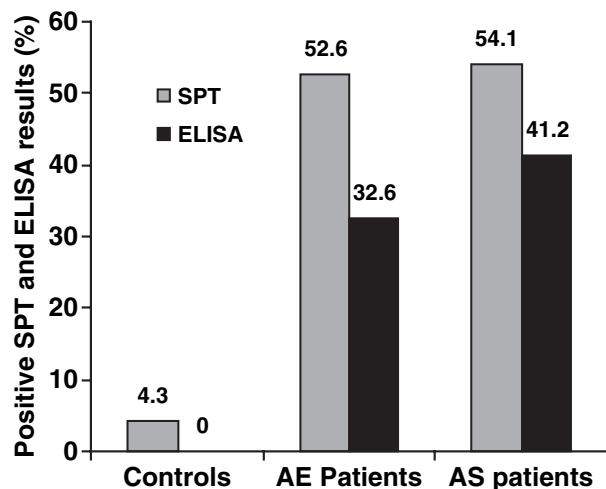


Figure 1 Comparison of positive skin prick test (SPT) and ELISA (specific IgE) results among patients with atopic eczema (AE), asthma (AS) and healthy controls.

Table 1 Results of positive skin prick, ELISA and IgE-immunoblotting tests to *Candida albicans* allergens with respect to age and gender characteristics of patients and healthy controls

Characteristics	No. (%) patients and control group								
				SPT (+)		ELISA (+)		IgE-Immunoblotting (+)	
	AE	AS	Control	AE	AS	AE	AS	AE	AS
Age									
<15	11 (11.6)	4 (4.7)	6 (8.6)	5 (45.5)	2 (50)	4 (36.4)	2 (50)	4 (36.4)	2 (50)
15–24	24 (25.3)	6 (7.1)	12 (17.1)	15 (65.5)	5 (83.3)	9 (37.5)	5 (83.3)	10 (41.7)	3 (50)
25–34	26 (27.4)	20 (23.5)	18 (25.7)	14 (53.8)	10 (50)	9 (34.6)	7 (35)	12 (46.2)	8 (40)
35–44	24 (25.3)	27 (31.8)	19 (27.1)	11 (45.8)	12 (44.4)	8 (33.3)	9 (33.3)	11 (45.8)	12 (44.4)
45–54	7 (7.4)	16 (18.8)	9 (12.9)	4 (57.1)	7 (43.8)	0 (0)	6 (37.5)	0 (0)	7 (43.8)
≥55	3 (3.2)	12 (12.6)	6 (8.6)	1 (33.3)	10 (83.3)	1 (31)	6 (50)	1 (33.3)	10 (83.3)
Total	95	85	70	50	46	31	35	38	42
Gender									
Male	22 (23.2)	40 (47.1)	25 (35.7)	10 (45.5)	24 (60)	3 (13.6)	13 (32.5)	6 (27.3)	22 (55)
Female	73 (76.8)	45 (52.9)	45 (64.3)	40 (54.8)	22 (48.9)	28 (38.4)	22 (48.9)	32 (43.8)	20 (44.4)

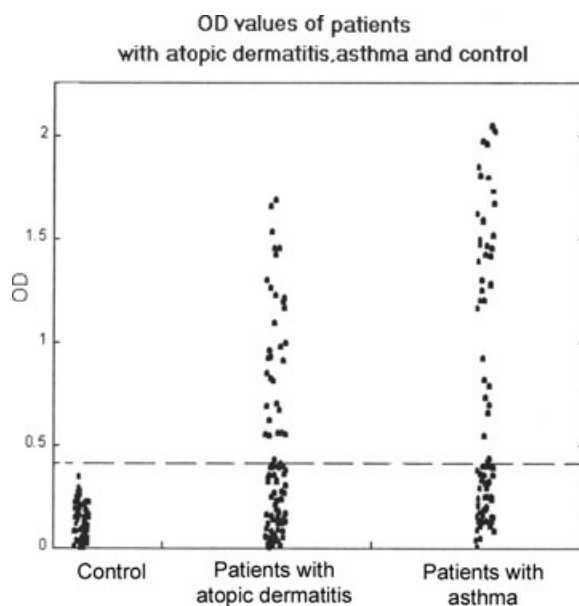
Three (4.3%) of healthy controls had positive reactions in skin prick test.

No positive results of ELISA and IgE-immunoblotting tests were observed in healthy controls.

AE, atopic eczema patients; AS, asthmatic patients; SPT, skin prick test.

ELISA test

The mean OD (405) + 3 SD control group was 0.408 (Fig. 2). Positive sera were defined with OD >0.408. Concerning the above mentioned point, of 95 patients with AE and 85 AS patients, 31 (32.6%) and 35 (41.2%) had demonstrated ELISA positive results, respectively, whereas no positive results were obtained in individuals of the healthy group (Fig. 1). Significant

**Figure 2** The OD values of patients with atopic eczema, asthma and healthy controls.

differences were observed between patients (AE and AS patients) and healthy controls ($P < 0.05$). Females with AE had significantly more serological positive results to *C. albicans* antigens than males ($P < 0.05$), but these differences were not significant between patients with respect to gender (in patients with AS), age, familiar history, and AE or AS diseases (Table 1). In addition, with respect to the results of SPT and ELISA tests, there were no significant differences in the severity of AE (SCORAD index) among three groups.

IgE-immunoblotting

For further identification of IgE-binding components of the allergenic extract of *C. albicans*, immunoblot was performed with each serum. Of 95 sera obtained from patients with AE sera and 85 sera from AS patients, 38 (40%), and 42 cases (49.4%) showed positive results, respectively, but all of the healthy controls (100%) were negative (Table 1). There were significant differences between patients and healthy controls ($P < 0.05$), whereas no significant difference was observed between the two patient groups. IgE immunoblots of the patients with AE and AS are presented in Figs 3 and 4, respectively.

The IgE responses were directed against 33 different bands in patients with AE and AS patients (Table 2). Three major allergens were found in patients with AE: 25 (76.3), 34 (50) and 57 kDa (42%), whereas in AS patients, 22 (59.5), 25 (59.5), 32 (45.2) and 34 kDa (66.7%) bands were major reactive allergens.

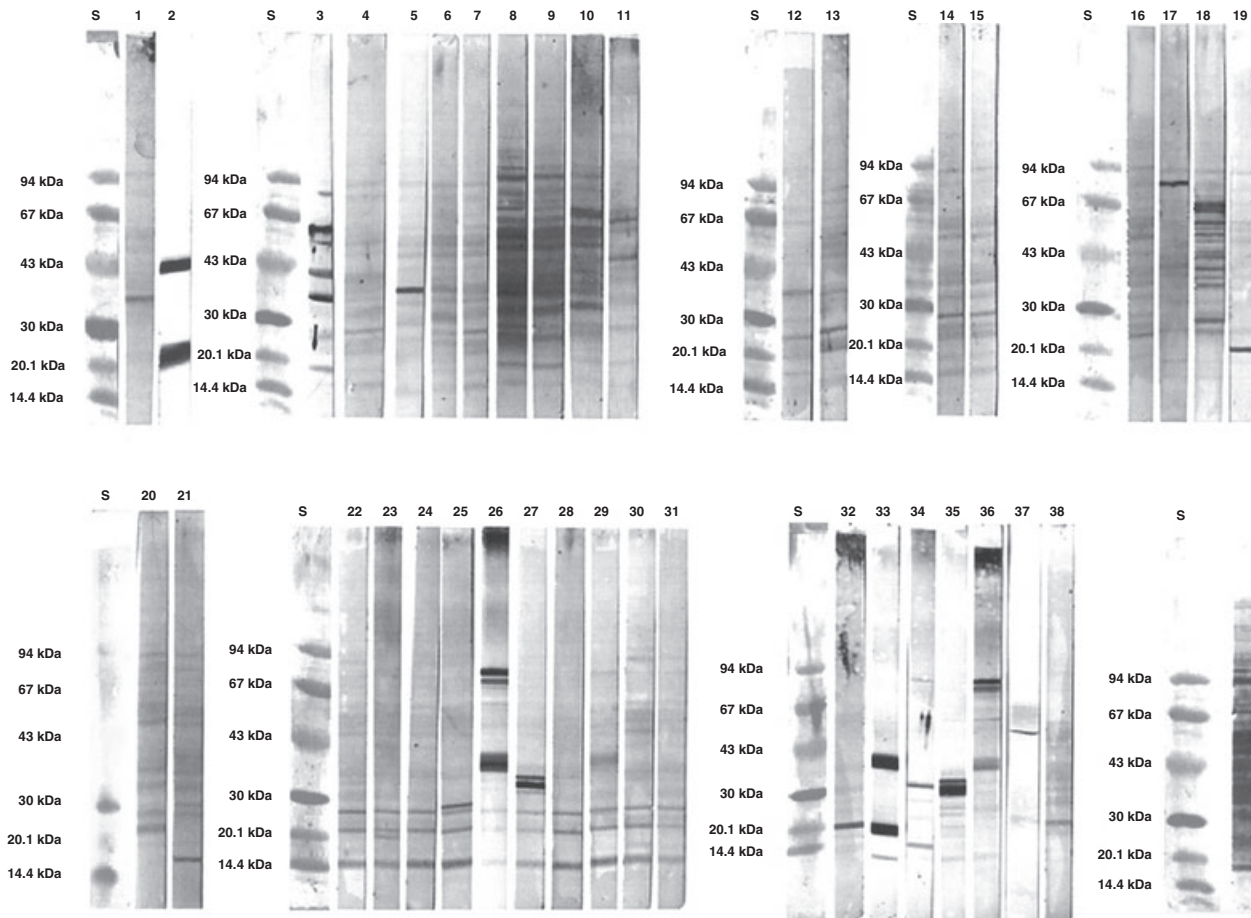


Figure 3 IgE binding to *Candida albicans* allergenic components in immunoblotting experiments with 38 sera of patients with atopic eczema.

Discussion

In recent years, interest in investigating the role of sensitisation to fungal allergens in different allergic diseases and in determining the exact sensitisation pattern of each patient has increased. *Candida albicans* is associated with bronchial asthma, allergic rhinitis, AE, chronic wheal and other allergic symptoms. In this study, 52.6% and 54.1% of patients with AE and AS had positive reactions to *C. albicans* antigens in SPT, respectively. In a study of 117 adult patients with AE, 28 patients with respiratory allergy and 39 healthy controls, 52% of patients with AE were SPT positive to *C. albicans* extract compared to 22% of patients with respiratory allergy.⁵ These findings are in accordance with other reports.^{26,27} It is suggested that some factors such as various extraction methods and different criteria to select the patients and protocol of SPT can affect the SPT results. Despite the other findings,²⁸ no significant differences were observed in SPT results between

patients with respect to gender, age, and familiar history of atopy in our study. In general, positive results to *C. albicans* antigens were reported between 30% and 70% in SPT.^{4,5,29–31} In a study conducted by Khosravi *et al.* [32] 41.4% of AE patients, who had other allergic disorders, showed positive reactions to *Malassezia furfur* antigens. In this study, 38.9% of AE patients had also other allergic disorders. These findings indicate that other allergic disorders do not prevent patients from becoming sensitised to *C. albicans* and *M. furfur* allergens. Interestingly, 77.6% of AS patients with positive SPT to *C. albicans* antigens reacted with other standard allergens such as pollens and mites.

In this study, ELISA positive results were obtained in 32.6% and 41.2% from AE and AS patients, respectively and incidence rate is similar to the rate obtained by Doekes *et al.* [11], Lindgren *et al.* [28], Back *et al.* [33], Okudaria *et al.* [34] and Gumoski *et al.* [35]. In another report by Nermes *et al.* [36] of 78 patients with AE and of 30 patients with AS, 53 (68%) and 16 (53%) cases

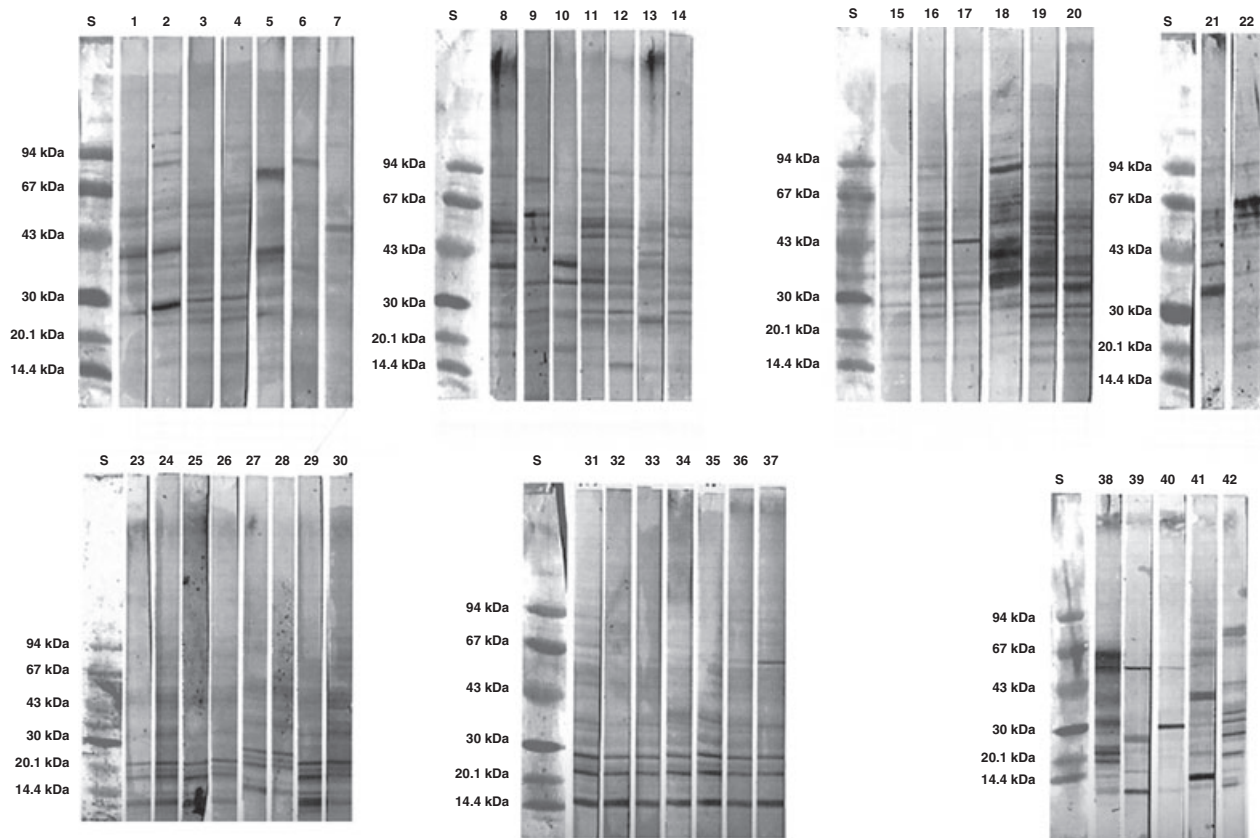


Figure 4 IgE binding to *Candida albicans* allergenic components in immunoblotting experiments with 42 sera of patients with asthma.

Table 2 The major and intermediate allergenic components of *Candida albicans* in patients with atopic eczema (AE) and asthma (AS)

Patient	<i>Candida albicans</i> allergens	
	Major allergens (kDa, %)	Intermediate allergens (kDa, %)
AE	25 (76.3), 34 (50), 57 (42)	16 (31.6), 20 (39.5), 22 (34.2), 23 (34.2), 25 (76.3), 30 (39.5), 32 (34.2), 34 (50), 53 (34.2), 57 (42), 94 (31.5)
AS	22 (59.5), 25 (59.5), 32 (45.2), 34 (66.7)	16 (38), 18 (33.3), 22 (59.5), 25 (59.5), 32 (45.2), 34 (66.7), 46 (30.9), 57 (35.7), 94 (38.1)

showed elevated IgE levels to *C. albicans* compared with healthy controls, respectively. Kimura *et al.* [37] found specific-*Candida* IgE antibodies in 87% of patients with AE compared to only 15% in patients with AS. The frequencies of serum specific *Candida* IgE levels are different from one study to another. In general, the number of patients with IgE antibodies was larger for adults than for children, for patients with severe AE than for those with mild AE, and for patients with a high total IgE level. An explanation for the variations observed could be that different extraction procedures were used to prepare the extracts for testing. In respect of specific IgE responses, our study showed that

significant differences between females and males with AE. *Candida* colonisation is common in adult female and this could explain the gender differences in sensitisation because the immune system of colonised individuals is exposed to higher allergen levels than that of individuals subject only to environmental exposure. For this reason, the result of ELISA test was not significant with respect to gender in AS patients.

Candida albicans extracts have different allergenic components, which induce allergic reactions in some atopic patients. Various authors reported different antigens, as allergens, in different patients and geographical locations.¹⁶ In our study, 33 of 43 protein

bands with molecular weights ranging from 13 to 124 kDa had reacted with IgE antibodies. In AE patients; 25, 34, and 57 kDa bands were predominant allergens, whereas in As patients; 22, 25, 32, and 34 kDa bands were recognised as major allergens. Ishiguro *et al.* [10] have identified different allergens (175, 125, 46, 43, and 37 kDa) from *C. albicans* that reacted with IgE antibodies from sera of 57 patients with allergic diseases. Savolainen *et al.* [4] found the most dominant protein allergens (27 and 46 kDa) from *C. albicans*. In two other studies, the major antigens, which showed the highest IgE-binding frequency in allergic patients, were 40 and 46 kDa proteins that reacted with 77% of the serum samples.^{4,6} In this study, we did not detect 40 kDa protein as reactive allergen, but 30.9% of patient's sera had positive reaction to 46 kDa allergen. This might show that the antigens vary in immunogenicity in humans. As allergic disorders are dependent on environmental and genetic factors, immune responses to the allergens may vary independent of population and race. Therefore, standardisation of the fungal extracts is imperative to improve the diagnosis of fungal sensitisation.

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