

Allergenic components in three different species of *Penicillium*: crossreactivity among major allergens

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Summary

Background *Penicillium* species have been considered as important causative agents of extrinsic bronchial asthma. However, little is known about the allergens of these ubiquitous airborne fungal species.

Objective This study compares the allergenic profiles and allergenic crossreactivity among allergens of three prevalent airborne *Penicillium* species.

Methods IgE-binding *Penicillium* components were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)-immunoblotting using sera from 67 asthmatic patients. The presence of allergenic crossreactivity was analysed by immunoblot inhibition.

Results Among the 67 serum samples tested, 15, 14 and 11 samples showed IgE reactivity to components of *P. citrinum*, *P. notatum* and *P. brevicompactum*, respectively. All 15 *P. citrinum*-positive serum samples showed IgE-binding to a 33 kDa extract component of this species. Thirteen (93%) of the 14 *P. notatum*-positive serum samples and 10 (91%) of the 11 *P. brevicompactum*-positive sera also showed IgE reactivity to components with a molecular weight of about 33 kDa in individual *Penicillium* species. All of the 10 *P. brevicompactum* 33 kDa component-positive serum samples showed IgE reactivity to the 33 kDa components of the other two *Penicillium* species tested. Dose-dependent inhibition of IgE-binding to these major allergens was observed when the positive serum sample was absorbed with different amounts of individual allergenic extract as well as with different amounts of extracts prepared from the other two *Penicillium* species.

Conclusion Although different allergenic profiles were observed in the three different *Penicillium* species tested, results showed that there was an IgE crossreactivity among the 33 kDa group major allergens of *P. citrinum*, *P. notatum* and *P. brevicompactum*.

Keywords: allergens, *Penicillium* species, immunoblotting, IgE crossreactivity

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Introduction

Mould spores have long been considered as causative agents for extrinsic bronchial asthma [1]. Licorish *et al.* have further confirmed that *Alternaria* and *Penicillium* spores in relatively natural states and numbers were potent

immunopathogens for asthma [2]. More than 150 different species have been discovered in the genus *Penicillium* [3]. However, the allergenic relationship among different *Penicillium* species and therefore the species recommended for clinical use are not yet identified. Muilenberg *et al.* reported that the five most frequently recovered species of *Penicillium* in and out of doors in Topeka KS were *P. spinulosum*, *P. brevicompactum*, *P. oxalicum*, *P. chrysogenum* (*P. notatum*) and *P. citrinum* [4]. However, the three most prevalent *Penicillium* species in Taipei area isolated by us were

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P. citrinum, *P. crustosum* and *P. implicatum* [5]. To understand whether there were different allergenic profiles in different *Penicillium* species, components recognised by human IgE antibodies in three different *Penicillium* species (*P. citrinum*, *P. notatum* and *P. brevicompactum*) were analysed by immunoblotting. The presence of IgE cross-reactivity among allergens of different *Penicillium* species was also analysed by immunoblot inhibition. Results obtained showed the presence of allergenic crossreactivity among the 33 kDa-group major allergens of the three different *Penicillium* species tested.

Materials and methods

Serum samples

Sixty-seven serum samples from patients with bronchial asthma attending allergy clinics at the Cathay General Hospital, Taipei were collected. Patients were selected on the basis of reported history of asthma. After collection, all the serum samples were stored in aliquots at -70°C .

Allergenic extracts

Among the three *Penicillium* species tested, only the extract of *P. notatum* was commercial available and the extract of this *Penicillium* species (Item no. M19) obtained from Greer Laboratories (Lenoir, North Carolina, USA) was used in the present study. For the preparation of allergenic extracts of *P. citrinum*, fungal mats of *P. citrinum* strain 52-5 isolated from the air of Taipei area [5] containing both spores and mycelia were collected after its growth in a liquid medium containing Czapek concentrate [3] (10 mL/L), K_2HPO_4 (1.0 g/L) and sucrose (30 g/L) for 3 weeks at room temperature. For preparation of the extract of *P. brevicompactum*, ground and freeze-dried preparation of this *Penicillium* species was obtained from Allergon AB, Engelholm, Sweden. The allergenic extracts of both *P. citrinum* and *P. brevicompactum* were prepared by extracting the fungal mats (1:5, v/v) with phosphate-buffered saline (PBS) (containing 85 mmol/L of NaCl, 2.6 mmol/L of KH_2PO_4 and 49 mmol/L of Na_2HPO_4 , pH 8.0) for 36 h at 4°C with constant shaking. After centrifugation, the supernatant was dialysed against 50 mmol/L of ammonium bicarbonate for 48 h at 4°C . After sterile filtration (0.2 μm ; Sartorius, Hayward, California, USA), the extract was lyophilised in aliquots and stored at 4°C . The protein content of the extract was determined by the dye-binding method of Bio-Rad (Richmond, California, USA).

Immunoblot analysis

The immunoblot analysis of components of different *Penicillium* species recognised by human IgE antibodies was performed essentially as described previously [6,7]. Components of individual allergenic extract in SDS-PAGE sample buffer (containing 2% SDS and 5% 2-mercaptoethanol), which were boiled for 5 min, were then separated on a 12.5% SDS-polyacrylamide gel and transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes (0.45 μm , Millipore, Bedford, Mass. USA). After being blocked with 1% skim milk in Tris-buffered saline (TBS, 20 mmol/L of Tris, 500 mmol/L of NaCl, pH 7.5), the blots were incubated with diluted serum samples (1:5 dilution in TBS containing 0.05% Tween 20 [TBST] and 1% skim milk) for 16 h at 4°C . After washing with TBST, the blots were incubated with alkaline phosphatase-conjugated monoclonal anti-human IgE antibodies (Pharmingen, San Diego, California, USA) for 1.5 h at room temperature. Following washing procedures, the blots were developed in a substrate solution of carbonate buffer (0.1 M NaHCO_3 , 1.0 mM MgCl_2 , pH 9.8) containing 0.35 M BCIP (5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt) and 0.37 M NBT (p-nitro blue tetrazolium chloride) for about 30 min. After washing with water, the deposition of the purple stain which demonstrated antigen-antibody binding was recorded by photography. For control, serum samples obtained from patients allergic to house dust mite only were used.

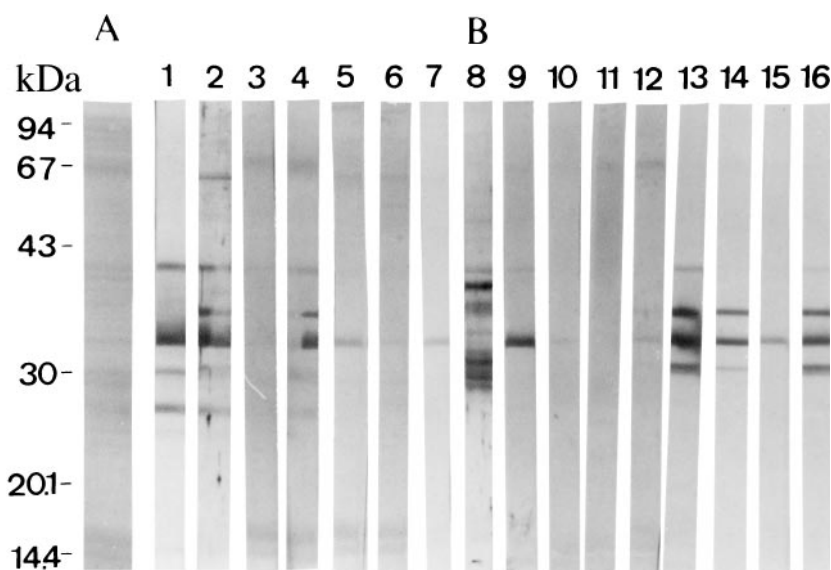
Immunoblot inhibition

To test the presence of allergenic crossreactivity among allergens of different *Penicillium* species, immunoblot inhibition was performed. An IgE-positive serum sample was mixed individually with different amounts (100 μg or 300 μg) of the three different *Penicillium* extracts by end over end rotation at 4°C for 1 h. The PVDF blots were then incubated individually with the IgE-positive serum or with different serum-extract mixtures at 4°C for 16 h. For control, the blots were incubated with the same positive serum sample which has been mixed with different amounts (100 μg or 300 μg) of an allergenic extract of *Candida albicans* prepared as described before [8]. The blots were washed and incubated with alkaline phosphatase-conjugated monoclonal anti-human IgE antibodies and developed with the enzyme substrate as described above.

Results

To identify IgE-binding components in extracts of three different *Penicillium* species, immunoblot was performed. Among the 67 sera tested, 16 (24%) showed

Fig. 1. IgE-binding components of *P. citrinum* analysed by SDS-PAGE-immunoblot. Panel A shows the Coomassie blue-stained protein profile of the extract of *P. citrinum* on the PVDF blot. The 16 strips in panel B show the result of immunoblot by using 16 different serum samples obtained from asthmatic patients.



positive results (Figs 1–3). Among these 16 positive samples, 15, 14 and 11 showed IgE-binding to components of *P. citrinum*, *P. notatum* (obtained from Greer Lab., Inc., Item no. M19) and *P. brevicompactum*, respectively (Figs 1–3). All 15 *P. citrinum*-positive sera showed IgE-binding to the 33 kDa component of *P. citrinum* (Fig. 1 and Table 1).

Thirteen (93%) of the 14 *P. notatum*-positive and 10 (91%) of the 11 *P. brevicompactum*-positive samples showed IgE-binding to the 33 kDa components of individual *Penicillium* species (Figs 2 and 3 and Tables 2 and 3). These 33 kDa components may thus be considered individually as a major allergen of each *Penicillium* species tested. All of

Table 1. Components of *P. citrinum* recognized by IgE antibodies in sera of asthmatic patients analysed by immunoblotting

Serum no.	Molecular weight (kDa) and frequency of IgE-binding ⁺											
	61 (7%)	47 (7%)	40 (53%)	38 (7%)	35.5 (47%)	33.5 (7%)	33 (100%)	31.5 (7%)	30.5 (47%)	29 (7%)	28 (7%)	26 (20%)
1			+				+		+			+
2	+		+		+		+		+			+
3			+				+					
4			+		+		+		+			+
5							+					
6							+					
7							+					
8		+	+	+	+	+	+	+	+	+	+	
9			+				+					
10							+					
11*												
12					+		+					
13			+		+		+		+			
14					+		+		+			
15							+					
16			+		+		+		+			

⁺ The frequency of IgE-binding (%) was calculated by dividing the individual number of positive serum sample(s) by the total positive number of 15 in the present immunoblotting. *Represents the serum sample which showed negative result in the present immunoblotting.

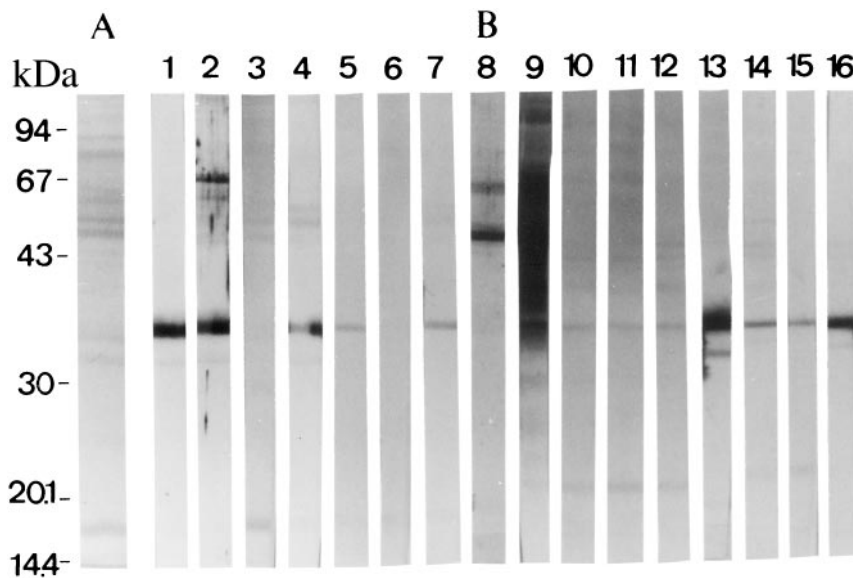


Fig. 2. IgE-binding components of *P. notatum* analysed by SDS-PAGE immunoblot. Panel A shows the Coomassie blue-stained protein profile of the extract of *P. notatum* (obtained from Greer) on the PVDF blot. Strips in panel B show the result of immunoblot by using the same 16 serum samples as those shown in Fig. 1.

the 10 serum samples which showed IgE-binding to the 33 kDa component of *P. brevicompactum* reacted with the 33 kDa major allergens of the other two different *Penicillium* species.

In addition to the 33 kDa major allergen, IgE-binding to another 11 components of *P. citrinum* ranging in MWs from about 61–26 kDa was also observed (Fig. 1 and Table 1). Components with molecular weights of about 40, 35.5 and 30.5 kDa which showed frequencies of IgE-binding of 53% (40 kDa) and 47% (35.5 and 30.5 kDa) (Table 1), respectively may also be considered as important allergens of *P. citrinum*. Three (20%) of the 15 sera showed IgE-binding to the 26 kDa component of *P. citrinum*. Only serum no. 2 showed IgE-binding to the 61 kDa component of *P. citrinum*

(Fig. 1). The other six IgE-binding components of *P. citrinum* were recognised by IgE antibodies in serum No. 8 only as shown in Fig. 1.

In addition to the 33 kDa major allergen, the 32 kDa component of *P. notatum* (Greer Lab., Inc., Item no. M19) showed a frequency of IgE-binding of 36% (Fig. 2 and Table 2). The frequencies of IgE-binding of the other nine components of *P. notatum* were all less than 15% (Fig. 2 and Table 2). A similar finding was also observed for *P. brevicompactum*. In addition to the 33 kDa major allergen, components with molecular weights of about 30 and 36 kDa showed frequencies of IgE-binding of 45% and 27%, respectively (Fig. 3 and Table 3). The frequencies of IgE-binding of the other four components were all less than

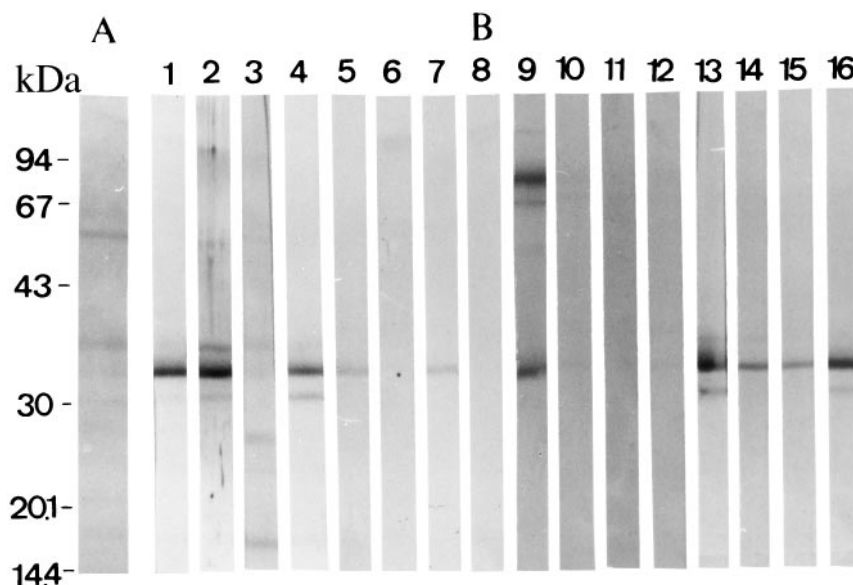


Fig. 3. IgE-binding components of *P. brevicompactum* analysed by SDS-PAGE immunoblot. Panel A shows the Coomassie blue-stained protein profile of the extract of *P. brevicompactum* on the PVDF blot. Strips in panel B show the result of immunoblot by using the same 16 serum samples as shown in Figs 1 and 2.

Table 2. Components of *P. notatum* recognized by IgE antibodies in sera of asthmatic patients analysed by immunoblotting

Serum no.	Molecular weight (kDa) and frequency of IgE-binding ⁺									
	>100 (7%)	68 (14%)	66 (14%)	64 (14%)	62 (14%)	60 (14%)	59 (14%)	49 (7%)	47 (14%)	33 (93%) 32 (36%)
1										+
2		+	+				+			+
3*										
4										+
5										+
6*										
7										+
8				+	+	+		+	+	
9	+	+	+	+	+	+	+		+	+
10										+
11										+
12										+
13										+
14										+
15										+
16										+

⁺ The frequency of IgE-binding (%) was calculated as described in Table 1. * Represents serum samples which showed negative results in the present immunoblotting.

Table 3. Components of *P. brevicompactum* recognized by IgE antibodies in sera of asthmatic patients analysed by immunoblotting

Serum no.	Molecular weight (kDa) and frequency of IgE-binding ⁺					
	>100 (9%)	80 (9%)	64 (9%)	36 (27%)	33 (91%)	30 (45%) 26 (9%)
1					+	+
2				+	+	+
3						+
4					+	+
5					+	
6*						
7						
8*					+	
9	+	+	+		+	
10*						
11*						
12*						
13				+	+	+
14				+	+	
15					+	
16					+	+

⁺ The frequency of IgE-binding (%) was calculated as described in Table 1. * Represents serum samples which showed negative results in the present immunoblotting.

10% (Fig. 3 and Table 3). It was observed that weak enzyme colour reactivity was sometimes detected for components in the 60–67 kDa and the 14–20 kDa areas when one of the cord serum samples was used. The weakly stained bands in those areas when sera from some asthmatic patients were used as shown in Figs 1 and 2 were not considered as positive IgE-binding in the present study. As a further control, serum samples obtained from mite-allergic patients showed negative result in the same immunoblotting (data not shown).

To test for allergenic crossreactivity among allergens of different *Penicillium* species, immunoblot inhibition was performed. For this study, more serum sample was collected from the patient whose serum was numbered No. 13 in Figs 1–3. In addition to showing IgE reactivity to all three major allergens, this patient also showed positive immediate skin reactivity to the extract of *P. notatum* obtained from Greer (Item No. M19). Strips A-a, B-a and C-a in Fig. 4 show the results of IgE-immunoblot of three different *Penicillium* species by using the serum sample obtained again from this patient which were similar to the results shown in strips B-13 in Figs 1–3. Dose-dependent inhibition of the immunoblot reactivity was observed when the PVDF blots of different *Penicillium* species were incubated with the same serum sample which had been mixed individually with different amounts of their respective allergenic extract (Fig. 4, strips b and c in panel A, d and e in panel B, f and g in panel C). A similar dose-dependent inhibition was also observed when different amounts of the other two *Penicillium* extracts were used as inhibitors as shown in strips d, e, f and g of panel A and strips b, c, d and e of panel C. In addition, inhibition of IgE-binding to the 33 kDa

component of *P. notatum* by different amounts of extracts from the other two different *Penicillium* species was also observed as shown in panel B, strips b, c, f and g. For control, dose-dependent inhibition of the IgE-immunoblot reactivity was not observed when the allergenic extract of *Candida albicans* was used (Fig. 4, strips h and i in panels A, B and C).

Discussion

Although fungal spores are well established as provokers of asthma and IgE-mediated allergy [1], there have been relatively few studies on fungal allergens as well as the allergenic crossreactivity between allergens of different fungal species. In the present study, the allergenic profiles of three prevalent air-borne *Penicillium* species were analysed by SDS-PAGE immunoblot using sera from asthmatic patients. The results showed that although different IgE-binding profiles were observed for different *Penicillium* species, components with molecular weights of about 33 kDa have been identified as a major allergen of each *Penicillium* species tested. The finding that there were allergens with similar molecular weights which could be found in several different species of a single genus has also been reported before [9]. For example, mite components with molecular weights of about 25 kDa and 14 kDa have been recognised as the group 1 and group 2 major allergens of two prevalent house dust mite species *D. pteronyssinus* and *D. farinae* [10,11]. Since the frequencies of IgE-binding of the 33 kDa group major allergens were all greater than 90% in each *Penicillium* species tested, it is worthwhile to

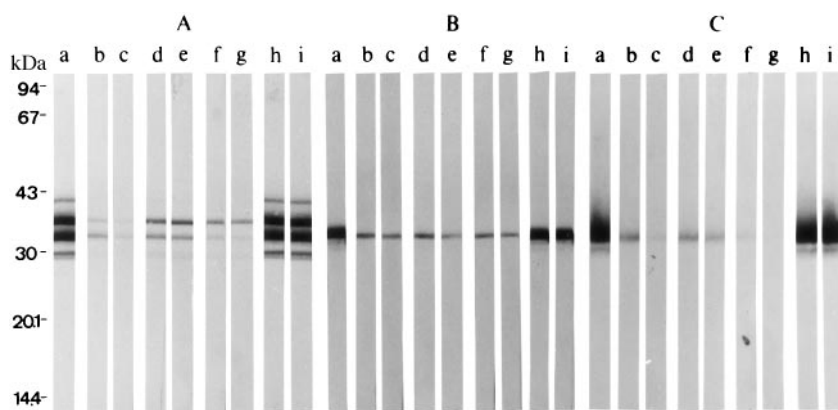


Fig. 4. Dose-dependent inhibition of the IgE-immunoblot reactivity of a positive serum sample to components of *P. citrinum* (panel A), *P. notatum* (panel B) and *P. brevicompactum* (panel C) by using different allergenic extracts. Strips a in panels A, B and C show the IgE-immunoblot reactivity of the serum sample used to three different *Penicillium* species tested. Strips b–i in all three panels represent results of immunoblot obtained by incubation of the PVDF blots with the same serum sample which has been mixed and incubated individually with 100 μ g (b, d, f, h) and 300 μ g (c, e, g, i) of *P. citrinum* (b and c of all three panels), *P. notatum* (d and e of all three panels), *P. brevicompactum* (f and g of all three panels) and *Candida albicans* (h and i of all three panels), respectively.

analyse if there are also corresponding 33 kDa allergens in other air-borne *Penicillium* species.

Results from immunoblot inhibition showed that there was IgE crossreactivity among the 33 kDa allergens of three different *Penicillium* species tested. The presence of allergenic crossreactivity between group 1 and group 2 major allergens of house dust mites has been well established before [9–11]. Allergenic crossreactivity among fungal allergens such as among the 14 kDa allergens of *Fusarium* species has also been reported recently [12].

In addition to the 33 kDa major allergen, immunoblot inhibition of IgE-binding to components of *P. citrinum* with molecular weights of about 40, 35.5 and 30.5 kDa by using extracts of different *Penicillium* species has also been observed. The finding suggests that there were shared allergenicity among these components of different *Penicillium* species. However, IgE-binding to components with molecular weights of about 40, 35.5 and 30.5 kDa was not all detected on blots of *P. notatum* and *P. brevicompactum*. The reason for this is not known. Similar findings were also observed in studies of crossreactivity between house dust and storage mites [13,14]. One possible explanation suggested by the authors is that the determinants in the negative extracts for some reason are blocked or destroyed during electrophoresis and/or electroblotting and therefore unavailable to the IgE antibodies [14].

In the present study, inhibition of IgE-binding to the 33 kDa group major allergens could be demonstrated by using extracts of all three different *Penicillium* species tested. Furthermore, according to the intensity of enzyme colour reactivity of immunoblots, results obtained also indicated that the degree of inhibition between *P. citrinum* and *P. brevicompactum* was greater than that between *P. notatum* and the other two *Penicillium* species. This finding suggests that *P. citrinum* may be allergenically more related to *P. brevicompactum* than to *P. notatum*. A related finding was also observed by us recently that a MoAb (55A) against the 33 kDa allergen of *P. citrinum* showed crossreactivity to the 33 kDa component of *P. brevicompactum* but not to the 33 kDa component of *P. notatum* (manuscript in preparation).

We have identified previously by SDS-PAGE immunoblot the components of *P. notatum* recognised by human IgE antibodies in sera from 19 allergic patients and 20 blood donors. Components with molecular weights of about 68 and 64 kDa with IgE-binding frequencies of 56% and 46%, respectively were recognised as important allergens of *P. notatum* [7]. The 33 kDa component showed a frequency of IgE-binding of 26% [7]. However, the allergenic extract of *P. notatum* used was prepared by extracting the ground and freeze-dried preparation of this fungal species obtained from Allergon AB, Engelholm, Sweden [7]. The extract of *P. notatum* used in the present study was obtained from

Greer, USA. In order to understand factors contributing to the discrepancy observed, 12 serum samples which showed IgE-binding to the 33 kDa component of *P. notatum* obtained from Greer were also used to identify IgE-binding components in the extract of *P. notatum* prepared by extracting materials obtained from Allergon, AB. Relatively weak IgE-binding to the 33 kDa component of *P. notatum* (Allergon AB) in only four of the 12 serum samples tested was observed (data not shown). Five of the 12 samples also showed relatively weak IgE-binding to components of *P. notatum* (Allergon AB) with molecular weights of about 68–64 kDa (data not shown). Thus, different extracts used may be considered as an important factor responsible for the discrepancy observed. It has been well documented that there was considerable variability in allergen content of extracts [15]. The difference in strains and serotypes of a single fungal species as well as variations in the preparation of these extracts may all contribute to variations observed [16,17].

In conclusion, results obtained from this study show allergenic crossreactivity among major allergens of three prevalent air-borne *Penicillium* species. This information will be useful in clinical diagnosis of *Penicillium* allergy. Furthermore, the discrepancy observed between identification of allergenic profiles by using different allergenic preparations also provided an evidence supporting the importance of standardisation of allergenic extracts used both clinically and for basic research.

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