

Impact of *Dermatophagoides pteronyssinus* mite body raw material on house dust mite allergy diagnosis in a Serbian population

L. BURAZER¹, K. MILOVANOVIC¹, M. MILOVANOVIC²,
O. VUCKOVIC¹, T. C. VELICKOVIC³
and M. GAVROVIC-JANKULOVIC³

¹Department of Allergy, Torlak Institute of Virology, Vaccines and Sera, Belgrade, Serbia, ²Department of Allergology, Zvezdara University Medical Centre, Belgrade, Serbia and ³Department of Biochemistry, University of Belgrade, Belgrade, Serbia

Abstract. House dust mite (HDM) allergy has different clinical and immunological patterns in different geographic regions. The impact of raw material of commercial *Dermatophagoides pteronyssinus* (Acari: Pyroglyphidae) mite bodies on the quality of allergen extracts for allergy diagnosis in the Serbian population has not been previously evaluated. House dust mite bodies obtained from manufacturers in Europe, South America and Australia were used in the preparation of allergen extracts for *in vivo* diagnosis and serological analysis in a group of 14 HDM-allergic adults. In the group of mite-allergic patients, there was no statistically significant difference in skin test reactivity (Wilcoxon matched pairs test) among the three HDM body extract preparations. In a CAP inhibition assay, two extracts (A and C) achieved maximum inhibition of >90%, whereas extract B demonstrated a different inhibition slope and lower inhibition potential (80%). However, a remarkable difference in immunoglobulin E reactivity using Western blot analysis with individual patients' sera was observed in one of the preparations (extract B). These findings emphasize the need for the careful selection of starting material for the preparation of HDM diagnostic reagents intended for use in patients from geographically distinct regions as these preparations can have implications on the selection criteria for patient-tailored immunotherapy of HDM allergy.

Key words. *Dermatophagoides pteronyssinus*, allergen extract, allergy diagnosis, house dust mites.

Introduction

The predominant species of house dust mite, of the genus *Dermatophagoides*, are a major cause of Type I hypersensitivity throughout the world (Tovey *et al.*, 1981; Bousquet *et al.*, 2007) and more than 50% of allergic patients are sensitized to this allergen source (Boulet *et al.*, 1997). Mites (Acarina) are divided into Pyroglyphidae, referred to as house dust mites (HDM), and Glycyphagidae, referred to as

storage mites (Nathanson, 1969; Wraith *et al.*, 1979; Iversen & Dahl, 1990). Since the mid-1960s, Pyroglyphidae mites have been established as the main source of house dust allergy (Voorhost *et al.*, 1964; Thomas & Smith, 1998) with *Dermatophagoides pteronyssinus* (European HDM) and *Dermatophagoides farinae* (American HDM) identified as the most important sources of aeroallergens in temperate climates (UCB Institute of Allergy, 1997; Bousquet, *et al.* 2004). Although it co-exists with *D. pteronyssinus* (Thomas *et al.*,

Correspondence: Dr Marija Gavrovic-Jankulovic, Department of Biochemistry, Faculty of Chemistry, University of Belgrade, Studentski trg 16, 11000 Belgrade, Serbia. Tel.: +381 11 3336 661; Fax: +381 11 2184 330; E-mail: mgavrov@chem.bg.ac.rs

2004), the Glycyphagidae mite *Blomia tropicalis* predominates in subtropical and tropical regions as a major allergen source (Fernandez-Caldas & Lockey, 2004).

House dust mite allergen extracts are made by aqueous extraction of a variable mixture of whole mite bodies, nymphs, faecal pellets, eggs and spent culture media (Eraso *et al.*, 1997; Batard *et al.*, 2006). The protein extract obtained is a very complex mixture of more than 30 proteins or glycoproteins, with up to 21 denominated allergen groups (Thomas *et al.*, 2002), shown to exhibit versatile biological functions (Custovic *et al.*, 1996). The major immunoglobulin E (IgE)-binding components of HDM extract have been reported for groups 1, 2, 3, 9, 11, 14 and 15, of which the most important are groups 1 and 2, which are present in high concentrations in household dust (Platts-Mills & Chapman, 1987; Best *et al.*, 2000; Weghofer *et al.*, 2008) and about 80% of patients have IgE antibodies to these two allergen groups. The proteolytic activity of HDM preparations has been attributed to Der p 1 (a cysteine protease), Der p 3 (a trypsin-like protease), Der p 6 (a chymotrypsin-like protease) and Der p 9 (a collagenolytic serine protease). In addition, group 4 revealed α -amylase activity, and groups 15 and 18 are chitinases (O'Neil *et al.*, 2006). The biological activity of the aforementioned allergens may compromise the quality of allergen extract preparations for diagnosis and immunotherapy. Moreover, excreted enzymes in mite faeces have been recognized as a major source of house dust allergens (Tovey *et al.*, 1981).

The quality of allergen products is a key issue in allergy diagnosis and therapy (Alvarez-Cuesta *et al.*, 2006). Although the use of recombinant HDM allergens in allergy diagnosis and immunotherapy is currently under validation (Pittner *et al.*, 2004; van Ree *et al.*, 2008), at present, routine *in vivo* diagnosis and immunotherapy are carried out using crude HDM extract preparations (Taketomi *et al.*, 2006) made from natural allergen sources. Depending on the manufacturer, purified mite body or whole culture material has been used for the commercial extract preparation, influencing the ratio between major secreted (i.e. Der p 1) and non-secreted (i.e. Der p 2) HDM allergens (Mayer *et al.*, 1994; van Ree, 2007), with a consequent impact on the quality of diagnostic products. To improve the diagnostic selection of patients for immunotherapy with Dp extracts, diagnostic tests based on major mite allergens (i.e. Der p 1, Der p 2) and highly cross-reactive mite allergens (Der p 10) have been proposed (Pittner *et al.*, 2004). Geographic variations in sensitization to environmental allergens have been observed (Bousquet *et al.*, 2007) and depend on climatic features (Levin, 2004). Because of the differential allergenicity to individual dust mite allergens, which has been linked to certain geographic regions (Cheong *et al.*, 2009), IgE reactivity to Dp extract in Western blot analysis has been proposed as a useful tool with which to design appropriate patient-tailored immunotherapeutic treatment.

The producer of allergy vaccines for the domestic market in Serbia, the Torlak Institute of Virology, Vaccines and Sera, employs a raw starting material of dust mite bodies for the production of allergy vaccines. Taking into consideration the differences in IgE reactivity in various geographic regions and the variable distribution of allergen variants in different

geographic environments (Piboonpocanun *et al.*, 2006), the aim of this work was to select the most potent allergen extract of three extracts prepared from commercial HDM bodies, in terms of its *in vitro* and *in vivo* IgE reactivity, for HDM allergy diagnosis in the Serbian population.

Materials and methods

Preparation of allergen extracts

Extracts of Dp were prepared from commercial HDM bodies of *D. pteronyssinus* purchased from three manufacturers [sample A: Allergon AB, Ängelholm, Sweden; sample B: National Bio-preparations Center (BioCen), Bejucal, Cuba; sample C: CSL Ltd, Parkville, VIC, Australia). Respective certificates of analysis declared 95% purity and 5% medium particles for sample A, approximately 99% homogeneous Dp species with <20% of remnant material from the spent culture medium for sample B, and only mites having the morphological characteristics of Dp for sample C.

Three protein extracts were prepared according to an identical procedure by overnight stirring of 5% (w/v) dried mite bodies in 150 mM phosphate buffer (pH 7.6) for 20 h at 4–8 °C. Extracts were clarified by centrifugation at 2000 g for 30 min. Supernatants were dialysed for 24 h at 4–8 °C in dialysis tubing (cut-off 5000 Da), filtered through a 0.22- μ m membrane (Pall Europe Ltd, Portsmouth, U.K.), and stored at –20 °C prior to analysis. Protein content was determined using the modified Lowry method (Bollag *et al.*, 1996), using bovine serum albumin (BSA) as standard.

Patients

Fourteen adult atopic patients (five men, nine women; mean age 36 years, range 18–59 years) referred to the Allergy Department of Zvezdara University Medical Center for HDM allergy, without prior record of immunotherapy, were enrolled in the study (Table 1). The study was performed with the approval of the local ethics committee and informed written consent was obtained from all patients. Serum-specific IgE to Dp extract (d1; Phadia AB, Uppsala, Sweden) was assayed using an automated immunofluorometric method (ImmunoCAP 100; Phadia AB). Results were expressed as CAP scores in classes 0–6, according to the manufacturer's instructions.

Skin prick testing

Skin prick testing (SPT) was performed with a standard battery of glycerinated extracts [grass, tree and weed pollen, house dust, bed linen dust, animal hair, mould, bacteria, bee venom, wasp venom, hornet venom, cockroach (all from the Torlak Institute of Virology, Vaccines and Sera)] and the three *D. pteronyssinus* glycerinated extract preparations from mite bodies (A, B, C) purchased from three manufacturers. Histamine phosphate at 1 mg/mL and phosphate-buffered saline (PBS) were used as positive and negative controls,

Table 1. Clinical and serological characteristics of the study population.

Patient no.	Sex/age, years	Co-sensitization	Disease, symptoms	Wheal/flare, mm	Wheal/flare, mm	Wheal/flare, mm	Wheal/flare, mm	Specific IgE, kUA/L
				Histamine	Extract A	Extract B	Extract C	
1	M/45	Tree, weed pollen	Pollinosis, urticaria	3/8	6/25	4/20	7/30	ND
2	F/59	Grass pollen	Rhinitis, pollinosis	5/8	5/12	6/15	4/15	ND
3	M/43	Grass pollen	Asthma	4/15	5/15	5/7	6/20	Class 3 3.77
4	F/41		Rhinitis, urticaria	4/15	5/25	6/20	9/25	Class 3 6.48
5	F/39		Asthma	3/10	8/20	10/30	6/30	Class 6 >100
6	F/20		Rhinitis	5/7	4/15	4/20	7/20	Class 3 17.70
7	F/49		Pruritis	5/20	9/35	5/35	9/50	Class 3 8.54
8	M/27		Rhinitis	4/35	4/30	4/20	3/30	Class 1 0.85
9	F/18		Rhinitis, asthma	3/3	6/20	4/9	9/25	Class 3 8.62
10	F/32		Rhinitis	4/15	5/17	5/16	4/22	Class 3 9.20
11	M/21	Grass pollen	Pollinosis, asthma	4/7	6/15	4/6	6/15	Class 3 11.40
12	F/38		Asthma	5/8	3/15	3/9	4/12	Class 3 5.43
13	M/37	Grass pollen	Rhinitis	4/10	9/27	6/25	8/30	Class 6 >100
14	F/37	Weed pollen	Rhinitis	4/7	6/8	7/19	6/14	Class 6 >100
15	F/48	[Control]		2/3	2/3	2/3	/3	Class 0 <0.35
16	M/48	[Control]		4/33	2/4	2/4	2/4	Class 0 <0.35
17	F/49	[Control]		5/12	2/3	2/3	2/3	Class 0 <0.35

ND, not determined.

respectively. The SPT results were evaluated after 20 min and a wheal measuring at least 3×3 mm was considered to indicate a positive result. Three non-atopic subjects were included as controls. The Wilcoxon matched pairs test was used for within-group comparisons of data obtained in SPTs with three different mite body extracts in the 14 HDM-allergic patients. $P < 0.05$ was considered significant.

SDS-PAGE and Western blot analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in line with the Laemmli method using an instrumentation apparatus with a discontinuous buffer system (Hoefer Scientific, Inc., San Francisco, CA, U.S.A.). Samples were boiled for 5 min before the run. Approximately 16 µg/mm of protein was resolved on a 12% gel at 80 V for 1 h and at 250 V for another 2 h. The gel was either stained with Coomassie Brilliant Blue R-250 (CBB) to visualize separated proteins, or resolved components were blotted using a semi-dry electro-transfer on to nitrocellulose membrane (0.45 µm) (Serva Electrophoresis GmbH, Heidelberg, Germany). The membrane was blocked in 20 mM Tris-buffered saline (TBS) containing 1% BSA and 0.1% Tween 20 for 1 h, and dried until development.

IgE detection

IgE-binding proteins were detected using Western blot analysis with individual patients' sera diluted five-fold, or with serum from a non-allergic individual in PBS containing 0.01% v/v Tween 20 (TPBS) with 0.1% BSA. Alkaline phosphatase-labelled monoclonal anti-human IgE (Sigma

Chemical Co., St Louis, MO, U.S.A.) was used as a secondary antibody. Binding patterns were visualized using a substrate solution of 1.5 mg BCIP (5-bromo-4-chloro-3-indolyl phosphate) (Serva Electrophoresis GmbH) and 3 mg nitro blue tetrazolium (NBT) (Serva Electrophoresis GmbH) in 10 mL of 100 mM Tris buffer, containing 150 mM NaCl, and 5 mM $MgCl_2$, pH 9.6, according to Harlow & Lane (1988).

CAP inhibition

The allergenic potentials of three mite body extracts were evaluated by competitive CAP inhibition assay with the ImmunoCAP System (Phadia AB). In brief, 120 µL of pooled patients' sera (CAP class 6) was incubated overnight at 4 °C with 120 µL of Dp extract A, B or C at increasing dilutions (0, 1 : 10, 1 : 100, 1 : 1000, 1 : 10000). Inhibited patients' sera (40 µL) were used for the quantification of specific IgE to d1 using the ImmunoCAP System (Phadia AB). A pool patient's serum without inhibitor was used as a positive control (test sample). The percentage of inhibition was calculated as $[1 - (OD \text{ of the inhibited sample} / OD \text{ of the test sample})] \times 100$ (where OD is optical density).

Results

SDS-PAGE, antigen and IgE-binding profile of Dp mite body extracts

Three Dp mite body extract preparations were analysed by SDS-PAGE. Staining with CBB revealed a protein pattern in the range of 10–116 kD for all three extracts. Predominant protein bands were observed at 116 kD, 40–67 kD, 25–30 kD

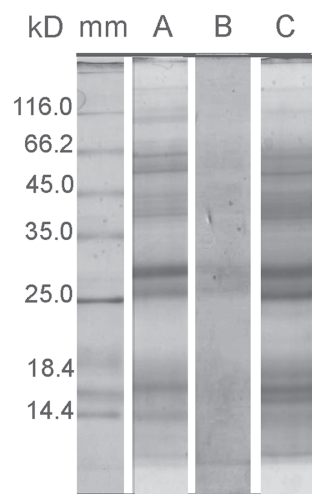


Fig. 1. SDS-PAGE analysis of *Dermatophagoides pteronyssinus* extracts A–C prepared from commercial mite bodies. mm, molecular mass markers.

and 12–18 kD. Extract B revealed a less complex protein profile than extracts A and C, with diffuse bands in the range of 35–45 kD (Fig. 1).

The 14 study subjects were used to evaluate the allergenic properties of three Dp mite body extracts. Six patients were co-sensitized to grass and/or weed pollen (Table 1). The comparison of IgE-binding profiles of Dp mite body extracts in Western blotting was performed using the sera of six individual patients, including three with specific IgE CAP class 6 and three with specific IgE CAP class 3 (Fig. 2). The IgE reactivity in Dp extracts was in the range of 10–116 kD, which correlates with results obtained by Tovey & Baldo (1985). Although not identical, extracts A and C showed similar IgE reactivity profiles, especially for allergens of lower molecular mass (12–35 kD). The difference was apparent for allergens of higher molecular mass in patients with high levels of specific IgE (patients 1–3). Strong IgE binding in patients with high specific IgE (patients 1–3) was shown for proteins of about 14–16 kD, presumably Der p 2 and/or Der p 5, in all three extract preparations. In addition, intense bands of 25 kD and 30 kD were present in extracts A and C, whereas there was almost a lack of IgE reactivity in this region in extract B (patients 1–3). A comparable pattern of IgE reactivity, but of lower intensity for extracts A and C, was obtained for patients with specific IgE class 3.

CAP inhibition

A pool of patients' sera with high specific IgE (CAP class 6) was incubated with serial dilutions of three Dp mite body extracts. Linear inhibition curves in Fig. 3 show inhibition of approximately 30–95%. Extracts A and C achieved maximum inhibition >90% (Fig. 3). The slopes of the inhibition curves for extracts A and C were almost parallel, which suggests that similar IgE-binding epitopes are present in these two allergen extract preparations. The different slope of the inhibition curve

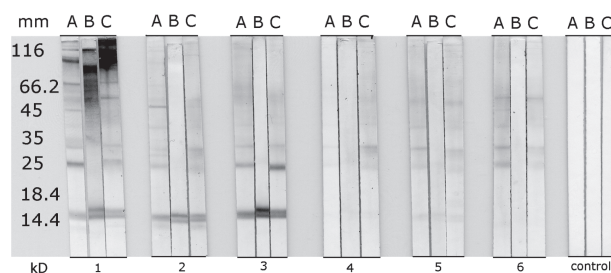


Fig. 2. IgE-binding profiles of three *Dermatophagoides pteronyssinus* extracts (A–C) obtained following Western blotting with sera of three patients with specific IgE CAP class 6 (patients 1–3) and sera of three patients with specific IgE CAP class 3 (patients 4–6). mm, molecular mass markers.

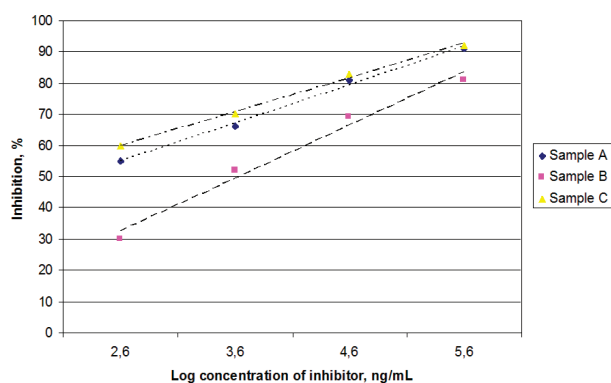


Fig. 3. CAP inhibition of IgE-binding by three *Dermatophagoides pteronyssinus* mite body extracts.

for extract B, as well as a lower inhibition potential, reflects an absence of the IgE-binding epitopes in extract B, presumably allergens in the range of 20–35 kD, but also some allergens of higher molecular mass, which may be relevant for the group of tested patients. Nonetheless, extract B revealed a maximum inhibition of IgE binding of 80%.

In vivo IgE reactivity

Skin prick tests with the three different Dp mite body extracts in the 14 HDM-allergic patients did not reveal any statistically significant differences (Wilcoxon matched pairs test). All three extracts were able to elicit a positive wheal reaction in all tested patients. The extracts also induced a wheal in non-allergic individuals. These individuals evidently had non-specific cutaneous hyper-reactivity, as demonstrated by the wheal and the flare in their histamine. Results are presented in Table 1 and summarized in Fig. 4. Spearman rank order correlations did not prove any positive correlations among tested extracts. Although allergens of low molecular mass (20–35 kD) in extract B were not recognized by patients' IgE, as proven by methods used in the biochemical characterization of the extracts, the SPT results showed comparable biological activity for three HDM-body preparations in our group of tested patients.

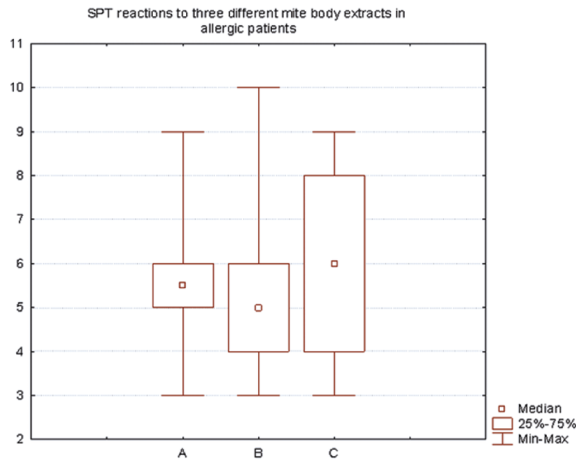


Fig. 4. Boxplots and whiskerplots of skin prick test reaction intensity (wheals in mm) to three different *Dermatophagoides pteronyssinus* mite body extracts in a group of 14 allergic adults.

Discussion

In order to make an accurate diagnosis, it is critical that the extract employed contains an adequate amount of all relevant allergen components. Although the quality of allergen extracts should be similar among different manufacturers, it has been acknowledged that this is difficult to achieve (Brunetto *et al.*, 2010). A recently conducted study which compared commercially available mite extracts for *in vivo* diagnosis revealed heterogeneous composition among mite extracts from different manufacturers (Brunetto *et al.*, 2010). Allergen extracts for *in vivo* diagnostics and immunotherapy intended for the local market, including the HDM extract, are produced on a small scale at the Torlak Institute of Virology, Vaccines and Sera. In this study, the impact of raw material on the quality of Dp allergen extracts prepared for diagnosis and therapeutic treatment in Serbian HDM-allergic patients was evaluated in order to facilitate the selection of the most potent/reactive allergen source for clinical applications. Certificates of analysis for raw materials do not include details on the cultivating conditions and the process of mite harvest, which can influence the quality and quantity of allergens in the HDM raw material. Because of possible variations in cultivating conditions, as well as the presence of allergen variants in geographically different environments, raw material of HDM bodies was purchased from three manufacturers located in Europe, South America and Australia, respectively. Allergen extracts were prepared according to the protocol authorized by the Torlak Institute of Virology, Vaccines and Sera. A group of 14 HDM-allergic adults participated in the study. The *in vivo* and *in vitro* methods employed suggest that, despite the inherent variability of the raw material of Dp mites, which is reflected in differences in allergen composition, SPT reactivity was comparable for the three extracts. However, a remarkable difference in IgE reactivity following Western blotting was observed in sample B. These results may be explained by the fact that different IgE responses observed in Western blotting reflect different allergen variants used for

testing, compared with the type of allergen variants involved in the sensitization of patients from our geographic region. For example, Der p 1 and Der p 2 showed frequent allelic variations which affect several amino acids (Piboonpocanun, *et al.*, 2006). Furthermore, variants of Der p 2 have different IgE-binding activities that can differ by 25–50% (Park *et al.*, 2002; van Ree 2002).

In accordance with previous studies (Tovey & Baldo, 1985, 1987), complex protein profiles were obtained for all three mite body extract preparations following SDS-PAGE. The IgE reactivity profiles were compared using sera from three patients with high levels of specific IgE (Class 6) and three patients with specific IgE scored as Class 3. In all three extracts, IgE was bound strongly by low molecular mass allergens (14–16 kD), probably representing Der p 2 and/or Der p 5 allergens. However, the difference in IgE reactivity in the range of 20–35 kD, which should correspond to Der p 6, Der p 7, Der p 8 and Der p 9, suggests an absence or low amount of those allergens, or their eventual modification, in extract B. It may also be a consequence of the variations caused by regional differences in mite allergens and sequence polymorphism (Thomas & Smith, 1998).

Pittner *et al.* (2004) reported the outcome of an analysis of IgE reactivity to nitrocellulose-blotted Dp allergens in a group of 208 mite-allergic patients, revealing two highly divergent reactivity profiles: profile A, with IgE reactivity exclusively represented by a 14-kD band, and profile B, characterized by IgE reactivity either to a great variety of allergens with molecular masses ranging from 14 kD to 200 kD, or reactivity to allergens with molecular masses outwith the 14-kD band. Interpretation of Western blotting results obtained with extract B would be misleading in the categorization of patients 2 and 3 (Fig. 2) as the analysis suggests that IgE from these patients reacted exclusively with a 14-kDa band. Such results could compromise decision making on patient-tailored therapeutic treatment by falsely selecting Der p 2 as an adequate therapeutic option. However, the difference observed in the IgE-binding profile with individual patients' sera following Western blot analysis was not as obvious in the competitive CAP inhibition assay, although inhibition curves suggest qualitative differences in the IgE-binding epitopes. It should be mentioned that IgE reactivity to some heat-labile mite allergens, such as Der p 1, or allergens present in low amounts, such as Der p 7, cannot be detected by IgE immunoblotting (Thomas & Smith, 1998; Pittner *et al.*, 2004).

The biochemical characterization revealed herein indicates that the extracts prepared from three different raw HDM materials are very different in protein content and composition. This difference was observed using *in vitro* tests (Western blot and CAP inhibition). *In vivo* testing showed that the SPT reaction intensity to all extracts was neither highly similar nor positively correlated. However, for the purpose of diagnosing HDM allergy based on SPT, any of the extract preparations can be employed in our group of patients. The diagnosis of HDM allergy was also confirmed in CAP by measuring levels of specific IgE. This result can be explained by the complexity of the protein content and the presence of various allergens in the extract. The lack or low amount of any of them did not prove to be an obstacle to diagnosing allergy to HDM. However,

it might be expected that, in a larger population of allergic patients, the presence of IgE in response to one of the missing allergens may be overlooked if extract B were employed as a diagnostic reagent. Although SPT reactivity in the patients in this study did not seem to be compromised by any of the three allergen preparations, because of the incorrect identification of the patient sensitization profile and the poor quality of the therapeutic preparation, immunotherapy with extract B could potentially be affected.

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