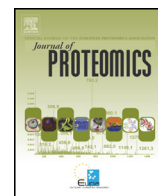




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Identification by serological proteome analysis of paramyosin as prominent allergen in dust mite allergy

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CRD

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WB

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western blot

ABSTRACT

Component-resolved diagnosis (CRD) of IgE-mediated hypersensitivities is challenged by the possibility that single patients are sensitized to components not commercially available to the clinical lab.

Here, we studied a patient with positive extract-based diagnosis of house dust mite (HDM) allergy based on routine *in vivo* (prick test) and *in vitro* (serum specific IgE) tests, whose serum scored negative for IgE to the three recombinant allergens routinely used in CRD (group 1 allergens, group 2 allergens and tropomyosin).

By means of serological proteome analysis via two-dimensional gel electrophoresis combined with immunoblotting and mass spectrometry, paramyosin (group 11 allergen: Der f 11 and Der p 11) was identified as the allergen component recognized by serum IgE from this patient in a raw allergen extract. Nine patients (64%) had IgE to Der p 11 in a group of 14 HDM allergic patients.

Biological significance: Our results add up to previous reports indicating that paramyosin is a clinically relevant HDM allergen and highlight that it can represent, in some patients, the first sensitizing component of this allergen source. This suggests that, at the moment, the use of allergen extract for the purpose of measuring IgE reactivity cannot be replaced by component resolved diagnosis and that group 11 allergens should be included among allergen components routinely tested in the clinical laboratory.

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1. Introduction

House dust mites (HDM), including *Dermatophagoides pteronyssinus* and *D. farinae*, are the most important perennial allergen sources in central Europe, which may cause allergic rhinitis as well as allergic asthma [1].

It was recently described that molecular diagnostics using the three commercially available major allergens Der p 1, Der p 2 and Der p 10 cannot detect HDM sensitization in a relevant number of cases [2]. Moreover, several reports have indicated widely divergent IgE reactivity patterns in HDM-allergic patients, highlighting that there is not one single major allergen component that can be used for diagnostic purposes worldwide [3–9].

We previously reported uncommon grass allergic patients who have a diagnosis based on a positive reaction to the relevant extract and a coherent clinical history but a negative score when tested with the set of allergen components presently available to the clinical laboratory [10].

Here, we describe a paediatric patient who came to our attention for being mite allergic, as confirmed by clinical evidence, prick testing and specific serum IgE determination performed with mite extracts. Serum IgE measurement yielded negative results for IgE to Der p 1, Der p 2, Der f 1, Der f 1 and Der p 10. Serological proteome analysis (SERPA), using his serum as a probe, allowed the identification of IgE reactivity toward paramyosin, a previously characterized mite allergen which is not included among allergen components routinely tested in the clinical laboratory.

2. Materials and methods

2.1. Patient's characteristics

A 8-years old boy with a clinical history of atopic dermatitis, had multiple sensitization to inhalants and oral allergy syndrome (patient

Abbreviations: CRD, Component-resolved diagnosis; HDM, house dust mite; SERPA, serological Proteome Analysis; WB, western blot.

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code: 0301). He had suffered atopic dermatitis from the age of two to the age of four. At the time of visit, no overt eczema was visible, but skin xerosis persisted. Prick test scored positive for *Phleum pratense*, *Dermatophagoides pteronyssinus* and *D. farinae* (ALK Abellö, Horsholm, Denmark) and negative for all other geographically relevant allergens that were tested (fagales, pellitory, ragweed, micophytes, animal danders). His mother reported that he suffered perennial rhinorrhea or nasal obstruction, which worsened during winter time, particularly in closed, heated environments. Springtime nasal symptoms which could be referred to grass sensitization were very mild. Thoracic auscultation did not reveal any abnormality. Specific IgE determination (ImmunoCAP, Thermo Fisher, Uppsala, Sweden) was requested, which yielded results shown in Table 1. This same serum was subsequently used as a probe for the evaluation of IgE reactivity to the mite proteome. For 2-DE analysis, control negative sera were obtained from non-atopic healthy subjects and from one patient allergic to grass pollen but not to HDM (patient code: 0108), while one serum from a subject strongly reactive with all tested HDM allergens (Der p1, Der p 2, Der f 1, Der f 2) (patient code: 0302) served as a positive control (Table 2). A set of HDM IgE positive patients' sera ($n = 14$), including those above reported, and a set of sera from patients with IgE to other allergens, including grass pollen, ($n = 11$) were used to test the frequency of rDer p11 recognition in HDM allergen-related or unrelated patients, respectively (Table 2).

The informed consent for the experimental use of sera was obtained from blood donors and was approved by the Ethics Committee of the IRCCS-San Raffaele Scientific Institute.

2.2. Allergen extract

The *Dermatophagoides farinae* raw extract used for the identification of IgE-reacting proteins, was purchased from Allergen/Thermo Fisher Scientific (Angelholm, Sweden).

2.3. Two-dimensional electrophoresis (2-DE)

2-DE was performed as described in ref. [11,12]. In brief, 200 µg of total protein extracts were applied to 7-cm IPG strips (pH 3–10 NL; GE-Healthcare) by in-gel rehydration. Focusing was performed with an IPGphor system (GE-Healthcare) (50 mA/strip maximum; gradient voltage, max 5000 V; 25 kVh total). Strips were reduced and alkylated by sequential incubation in equilibration buffer (50 mmol/l Tris-HCl, pH 8.8; 6 mol/l urea; 30% glycerol; 2% sodium dodecyl sulfate) containing 2% dithiothreitol or 2.5% iodoacetamide, respectively. Then, strips were transferred onto a 4–20% gradient or 12% sodium dodecyl sulfate polyacrylamide electrophoretic gels (SDS-PAGE) for the second-dimension separation on Mini-PROTEAN® electrophoresis cell (Bio-Rad). Preparative 2-DE gels for protein spot excision (see below) were generated by loading 700 µg of total protein. Preparative gels underwent to a partial protein transfer onto nitrocellulose membrane (i.e. 30 min at 80 V, 450 mA max), the edge of the gels were tracked on the membrane, then the gels were stained either with Coomassie colloidal blue (Sigma) according to the manufacturer, or MS-compatible silver staining [13]

Table 1
IgE reactivity of patient 0301.

Total IgE	65,3 ng/ml		
Specific IgE	<i>D. pteronyssinus</i> (extract)	ImmunoCAP (kU/l)	ISAC (IU/ml)
	<i>D. farinae</i> (extract)	0,13	na
	Der f 1	2,62	na
	Der f 2	na	0,00
	Der p 1	na	0,00
	Der p 2	<0.10	0,00
	Der p 10	<0.10	0,00

ImmunoCAP: Thermo-Fisher™; ISAC: immuno solid-phase allergen chip; na = not applicable.

and the corresponding membranes were incubated with the relevant sera (see below).

2.4. Western blot and dot blot analysis

Recombinant allergens Der p 11 and Der p 14 were produced as described in [14]. In brief, synthetic genes coding for Der p 11 and Der p 14 (GenBank accession number AY189697 and AF373221, respectively) with codons optimized for expression in *E. coli*, were synthesized with six histidine codons at the 3' end and inserted into plasmid pET-17b (Novagen). Allergens expressed in *E. coli* BL21(DE3) (Stratagene) were found in the inclusion body fraction and purified over Ni-NTA resin (Qiagen). Total proteins mite extracts or recombinant allergens Der p 11 and Der p 14 [14], recombinant Der f 2 (Indoor Biotechnologies) and recombinant Der p 23 (Biomay), resolved by 2-DE or SDS-PAGE, were transferred onto nitrocellulose membranes (2 h at 80 V, 450 mA max) and incubated overnight with patient sera (1:250 working dilution). For dot blot, recombinant allergens were directly spotted onto membranes. Immunoreactivity was revealed by incubation with polyclonal goat anti-human IgE, conjugated with horseradish peroxidase (Sigma), followed by electro-chemo-luminescence ECL™ Western Blotting Detection Reagent (GE-Healthcare) reaction and film exposure. Images from both preparative gels and the corresponding immunoblot were acquired at high resolution using a G:BOX Chemi XX6 System (Syngene, Cambridge, UK), then 2-DE protein patterns were analysed and matched in automated using Progenesis PG240 Same Spots software (Nonlinear dynamics, Newcastle, United Kingdom). Relative mass was estimated by comparison with reference markers (Precision, Bio-Rad) and isoelectric point values were assigned to the detected spots according to GE-Healthcare guidelines.

2.5. Protein identification by mass spectrometry analysis

Spots of interest were excised from gels, reduced, alkylated, and in-gel digested with bovine trypsin (Roche Diagnostics Corp) [15], then, liquid chromatography electron spray ionization tandem mass spectrometry (LC-ESI-MS/MS) analysis, performed as previously described [11]. In brief, 5 µl of digested sample were injected in a capillary chromatographic system (Agilent 1100 Series) equipped with a Nano Pump (Agilent) and peptide separation occurred in an RP C18 nano column. The eluting peptides were ionized by a nano-ESI online source and analysed on an API QStar PULSAR (AB-Sciex, Toronto, Ontario, Canada) mass spectrometer. Full-scan mass spectra (range m/z 350–1600) were collected, and for each spectrum, the 2 most intense doubly and triply charged ion peaks were selected for fragmentation. Tandem MS data files from chromatographic runs were combined and converted to MASCOT files using MASCOT.dll 2.2.07 through Analyst QS 1.1 (AB Sciex). All MS/MS samples were analysed using the MASCOT engine to search the Swiss Prot, Other Metazoa 2016_10 (10,510 sequences) database. Peptide mass tolerances of 200 ppm and 0.3 Da were used for precursor and fragment ions, respectively. The other search parameters were 2 allowed missing cleavages, oxidation of methionine as a variable modification, and carbamid methylation as a fixed modification.

3. Results

3.1. Proteomic identification of IgE reactive allergens

In order to identify the allergen recognized by IgE from patient 0301 we used a previously described approach based on the evaluation of reactivity to the relevant allergen proteome [10]. *D. farinae* was chosen due to the higher serological reactivity against total protein extract compared to *D. pteronyssinus*.

Four distinct protein spots (#1, 2, 3, and 4) of different molecular weight were recognized by patient's serum, but not by control healthy subject or patients allergic to grass pollen (Fig. 1). The protein spots

Table 2

Demographic features and serological IgE reactivity of allergic patients tested for rDer p 11 recognition.

		House dust mite													
Ptz #	Sex	Age (years)	Der p 11 ^a OD (a.u.)	Der p 23 ^a OD (a.u.)	Total IgE ^b (ng/ml)	D. pteronyssinus			D. farinae			Other allergens			
						Extract ^c (kU/l)	Der p 1 ^d (IU/ml)	Der p 2 ^d (IU/ml)	Extract ^c (kU/l)	Der f 1 ^d (IU/ml)	Der f 2 ^d (IU/ml)	Phl p 1 ^d (IU/ml)	Phl p 5 ^d (IU/ml)	Bet v 1 ^d (IU/ml)	Fel d1 ^d (IU/ml)
0301	m	8	21662	5367	63.3	0,13	<0.10	<0.10	2.60	na	na	19.33	0	0	0
0302	f	45	9048	9799	nd	31.56	5.78	3.96	21.23	4.00	11.90	0.31	0	0	0
0303	f	12	52158	8899	166.7	24.12	1.93	2.00	12.53	1.83	7.02	52.8	29.3	0	1.66
0304	f	9	11607	24361	nd	0	0	42.03	1.45	0	71.22	0	0	26.87	44.01
0305	f	35	41374	5023	357.12	56.75	48.16	32.70	56.53	57.40	128.51	41.83	0	141.97	116.89
0306	f	26	3469	4081	113.4	1.45	0	1.34	6.35	0	4.38	3.77	11.37	0	15.17
0307	m	22	7747	11221	42.45	9.53	0	2.20	9.00	0	11.91	10.47	0	0	8.23
0308	f	32	7297	10586	nd	1.45	18.55	97.68	0.55	52.01	14.00	35.13	27.36	0	0
0309	f	12	37739	8115	nd	0.98	2.20	5.47	2.45	46.00	26.49	1.49	0	0	7.99
0310	f	15	40237	6802	nd	0.88	0	6.00	19.45	0	31.74	0	0	0	0
0311	f	18	25493	7277	108.12	12.42	0.97	8.25	33.42	0.86	28.27	3.58	0	0	0
0312	m	19	59443	4904	172.12	3.56	0.54	1.26	40.52	0.34	3.91	13.78	10.18	0	39.14
0313	m	21	26946	3548	nd	5.45	0.89	1.46	5.45	4.42	5.53	28.04	15.94	21.19	17.83
0314	m	24	26593	4546	nd	3.67	0	11.43	7.56	0	41.98	2.53	1.82	24.43	0
0108	m	11	5721	5437	12.23	0	0	0	0	0	0	11.56	33.45	0	0
0109	f	23	2579	5533	nd	nd	0	0	0	0	0	43.91	29.59	0	0
0110	f	15	3294	5094	53.57	0	0	0	0	0	0	0.86	1.04	4.66	0
0111	f	46	3157	4438	nd	nd	0	0	nd	0	0	29.09	0.87	0	0
0112	m	32	4142	3664	nd	nd	0	0	nd	0	0	6.37	1.86	2.73	1.73
0113	f	45	2624	5154	nd	nd	0	0	nd	0	0	2.51	9.78	12.67	2
0114	f	27	3675	4821	nd	nd	0	0	nd	0	0	2.44	1.69	2.21	0
0115	m	45	4271	5207	nd	nd	0	0	nd	0	0	41.4	18.19	10.09	0
0116	f	12	10072	5315	43.12	0	0	0	0	0	0	9.39	0	0	0
0117	m	24	2847	4548	nd	nd	0	0	nd	0	0	22.83	15.13	0	0
0118	m	15	5237	5139	234	0	0	0	0	0	0	40.68	30.82	43.73	3.29

Der p 1, Der p 2 = *Dermatophagoides pteronyssinus* recombinant allergen p 1 and p 2; Der f 1, Der f 2 = *Dermatophagoides farinae* recombinant allergen f 1 and f 2; Phl p 1, Phl p 5 = *Phleum pratense* recombinant allergen p 1 and p 5; Bet v 1 = *Betula verrucosa* recombinant allergen v1; Fel d 1 = *Felis domesticus* recombinant allergen d1; na = not applicable; nd = not determined; f = female; m = male; OD = optical density; au = arbitrary units; grey boxes indicated positive sera for rDer p 11; = data obtained by western blot immunoreactivity against recombinant Der p 11 or Der p 23; = measured with total Ig ImmunoCAP (Thermo-Fisher™); = measured with D1 or D2 ImmunoCAP (Thermo-Fisher™); = measured with ISAC ImmunoCAP (Thermo-Fisher™).

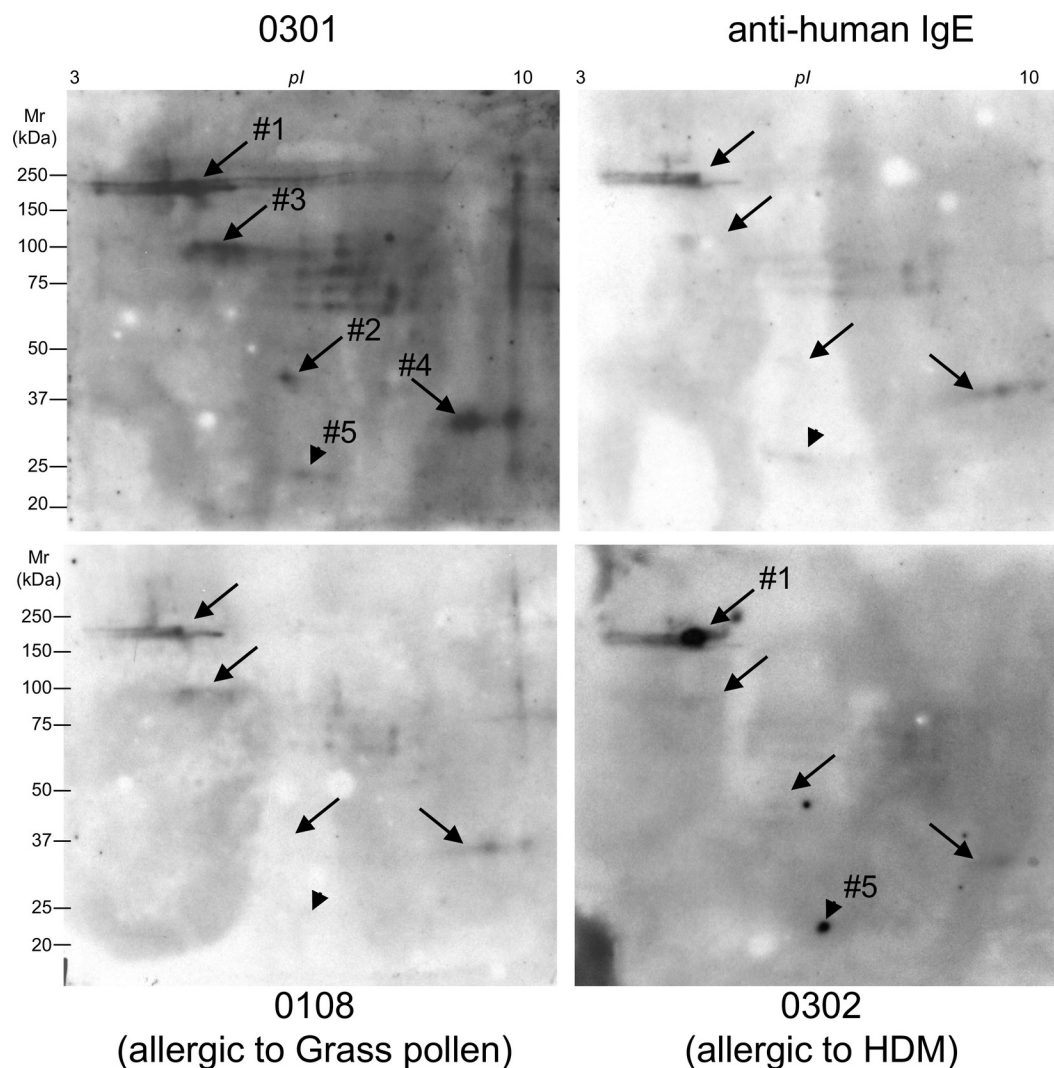


Fig. 1. Reactivity of patient 0301 serum to the *Dermatophagoides farinae* proteome. *D. farinae* extracts were resolved by 2-dimensional electrophoresis and then probed by Western-blot with sera ($n = 2$ technical replicates). IgE reactivity of patient 0301 serum was compared with the reactivity of an HDM-allergic patient (0302), with reactivity of a grass pollen allergic patient (0108) and the reactivity of the anti-human IgE secondary antibody as control. Arrows indicate the protein spots differently recognized by patient's sera, that underwent to mass spectrometry analysis for protein identification.

corresponding to IgE reactivity were excised from a preparative gel, and analysed by MS for protein identification.

The spot #2 with isoelectric point (pI) of 5.67 and relative mass (Mr) of 45.1 (kDa) gave the strongest and unique identification corresponding to the protein paramyosin, known as Der f 11 allergen [16].

Paramyosin was also identified in spot #3, that, compared to spot #2, likely representing a degradation product, showed a pI and Mr similar to the intact protein (Table 3).

Within spot #3 (Mr of about 107 kDa) two further proteins were identified, the peptidase 1 and the allergen Mag, known as Der f 1 and

Table 3
Protein identification by mass spectrometry.

Spot #	Protein name	Accession #	Allergen	pI/Mr (pH/kDa)		nLC-MS/MS		
				Theoretical	Experimental	Seq. cov. (%)	Frag. pep.	Mascot score
1	Peptidase 1	P16311	Der f 1	5.76/25.2	4.99/242.7	4	1	35 ^a
2	Paramyosin (Fragment)	Q967Z0	Der f 11	5.56/102.4	5.67/45.1	41	41	5507
3	Paramyosin	Q967Z0	Der f 11	5.56/102.4	5.26/107.4	13	9	470
	Peptidase 1	P16311	Der f 1	5.76/25.2	5.26/107.4	13	4	551
	Allergen Mag (Fragment)	P39673	Der f 14	6.92/39.6	5.26/107.4	19	7	355
4	Allergen Mag (Fragment)	P39673	Der f 14	6.92/39.6	7.43/34.1	19	7	119
5	Peptidase 1	P16311	Der f 1	5.76/25.2	5.75/24.6	13	4	381
Band	Protein name	Accession #	Allergen	Theor. Mr (kDa)	Exp. Mr (kDa)	Seq. cov. (%)	Frag. pep.	Mascot score
1	Mite group 2 allergen (DERF2)	Q00855	Der f 2	16.2	15.1	73	9	661

pI : isoelectric point; Mr : relative mass; kDa: kiloDalton; nLC-MS/MS: nano-liquid chromatography electron spray tandem mass spectrometry; Seq.cov.: sequence coverage; Frag. pep.: fragmented peptides.

^a The score was not significant, but identification was maintained since it represents the aggregate of the protein found in spot #5.

Der f 14, respectively [16]. In this spot, the identified proteins might represent a degradation product in the case of the Allergen Mag, a protein with predicted molecular weight (MW) of 190 kDa, and an aggregate/oligomer in the case of peptidase 1, a protein of 25 kDa (Table 3). Allergen Mag identification was also found by MS analysis in spot #4 corresponding to the predicted MW of the amino acid sequence deposited in the database, yet representing a fragment of a bigger protein.

Peptidase 1 was also the unique protein identified albeit with non-significant score, in the spot #1 likely representing macro-aggregates of about 240 kDa reacting with serum from patient 0301, but also partially reacting with control negative sera (Fig. 1 and Table 3). Notably, no IgE reactivity to rDer f 1 and rDer p 1 had been detected with the tests performed as part of the routine evaluation of patient 0301 in the clinical lab (Table 1).

We used the serum reactivity of a control patient (code: 0302) strongly reactive (>20 kU/l IgE) to both *D. pteronyssinus* and *D. farinae* (Der p 1, Der p 2, Der f 1 and Der f 2 allergens) to confirm the presence of peptidase 1 in the spots recognized by IgE from patient 0301. A strong reactivity of patient 0302 serum was found against spot #1, probably consisting in the macro-aggregates of peptidase 1, and against a spot of about 25 kDa (spot #5) (Fig. 1). MS analysis of spot #5 yielded the identification of peptidase 1 likely in its monomeric intact form as inferred by *pI* and MW similarity (Table 3). Almost no reactivity was observed for patient 0301 serum against the spot #5, suggesting that monomeric peptidase 1 was not targeted by IgE from this patient, a result that confirmed the absence of reactivity observed against group 1 recombinant allergens.

By 2-DE analysis was not observed a reactivity of patient 0302 serum against Der f 2, a protein of about 14 kDa. However, the validation of reactivity specific for Der f 2 was obtained by western blot analysis against both recombinant Der f 2 and *D. farinae* extract resolved by SDS-PAGE. A strong reactivity against rDer f 2 and against a band of about 15 kDa in the extract was observed for patient 0302 but not for patient 0301 serum (Fig. 2). The 15 kDa band from *D. farinae* extract was excised and analysed by MS yielding Der f 2 identification (Table 3). These results confirmed an absence of reactivity of patient 0301 to Group 2 allergens.

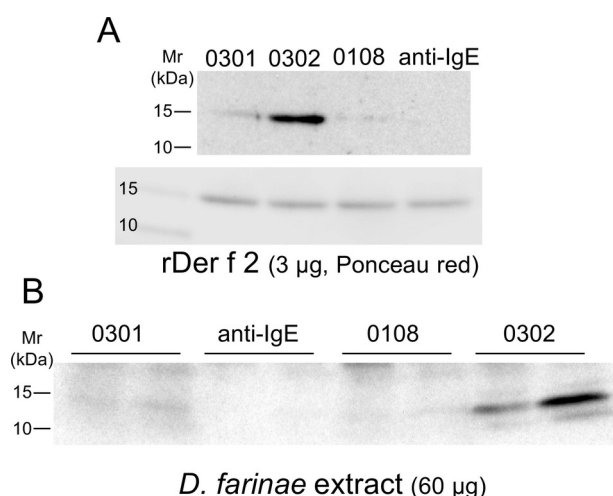


Fig. 2. Reactivity of patient 0301 serum against Der f 2 allergen. The IgE reactivity of patient 0301 serum was tested against (A) recombinant Der f 2 protein allergen ($n = 2$ technical replicates) and (B) *Dermatophagoides farinae* extract run in duplicate on the gel ($n = 3$ technical replicates), both resolved by SDS-PAGE. In panel A the bottom image shows Ponceau red staining of the recombinant allergen as control for gel loading. The reactivity was compared with the reactivity of an HDM-allergic patient (0302) positive for Der f 2, the reactivity of a grass pollen allergic patient (0108) and the reactivity of the anti-human IgE secondary antibody as control.

3.2. Validation of IgE reactivity to paramyosin (Der p 11) and allergen Mag (Der p 14)

In the absence of recombinant Der f 11, and due to its high homology with Der p 11 (97% sequence identity), His-tag recombinant Der p 11 expressed in *E. coli* [14] was used to test the reactivity of 0301 serum by western blot. Results showed IgE reactivity against both, the full Der p 11 recombinant protein and its degradation products reported to co-exist in the recombinant material (Fig. 3A) [14], which may explain the reactivity against the degradation products of Der f 11 observed in 2-DE experiment. On the contrary, no signals against rDer p 11 were observed with serum with unrelated IgE reactivity (code 0108, grass pollen reactive), nor with serum 0302, showing IgE reactivity level > 0.70 kU/l to HDM extracts, in agreement with the results of 2-DE analysis.

In a group of 25 sera of allergic patients, western blot evaluation scored positive for IgE to rDer p 11 in 9 sera (36%), considering the O.D. of the anti-IgE secondary Ab plus two standard deviation as cut off value. If only mite allergic patients, with IgE level > 0.70 kU/l to either or both mite extracts (*D. farinae* and *D. pteronyssinus*), were considered ($n = 14$) the percentage of sera positive for Der p 11 was 64% (9/14), while no reactivity to rDer p 11 was observed for sera of patients positive for other allergens (Fig. 3B and Table 2).

Der f 14 could be a potentially relevant allergen. However, it was not available as recombinant protein and the his-tag recombinant rDer p 14 molecule was used instead [14] (88% sequence identity by BLAST, P39673 vs. Q8N0N0 UniProtKB database entries) to check serum 0301 IgE reactivity by dot blot. This test yielded negative results (Fig. 4).

3.3. IgE reactivity recombinant Der p 23

Der p 23 has been recently identified as major mite allergen [17], but due to its low molecular weight (about 9 kDa) was not resolved in our 2DE. Therefore, in order to check whether patient 0301, in addition to Der f 11 and p 11, was reactive also to this major allergen, we tested his serum against recombinant Der p 23 resolved by SDS-PAGE. No reactivity was found for patient 0301, and for the 0108 grass pollen reactive patient. In contrast, Der p 23 was recognized by IgE from 0302 and 0304 HDM allergic patients, with 0304 showing a remarkable reactivity (Fig. 5A). In the group of 25 sera of allergic patients tested, western blot evaluation scored positive for IgE to rDer p 23 in 8 sera (32%), when the O.D. of the anti-IgE secondary Ab plus two standard deviation was established as cut off value. All sera with IgE to Der p 23 segregate in the group of mite allergic patients (8 out 14, 57%) (Fig. 5B and Table 2).

4. Discussion

House dust mites (HDM) are potent indoor allergen sources worldwide, which are associated to clinically relevant symptoms at different target organs, including the respiratory tract and the skin. According to available epidemiological studies, roughly one out of two allergic patients in Central Europe, who is living below the altitude of 1500 m is sensitized to HDM and suffers from symptoms and signs of allergic rhinitis, allergic conjunctivitis, allergic asthma and atopic dermatitis, singularly or more often in various association [18–20].

It has been reported that the allergen components from HDM allergen, which are routinely used in the clinical lab (namely, group I and II allergens, and tropomyosin), do not allow to diagnose mite allergy in a proportion of patients up to 16% [2]. This applies to different geographical areas, including Africa, Brazil, Europe, Asia (Korea and Thailand) and Australia, with different percentages affected not only by patients' genetics features but also by polymorphisms of HDM allergens [3–8,21, 22]. Becker et al. [2] by using a microarray based technology found that 5 out of 16 patients which scored negative for Der p 1, Der p 2 and Der p 10 had measurable IgE to Der p 23 (out of 11 components which were assayed), while Banerjee et al. [14] with a different

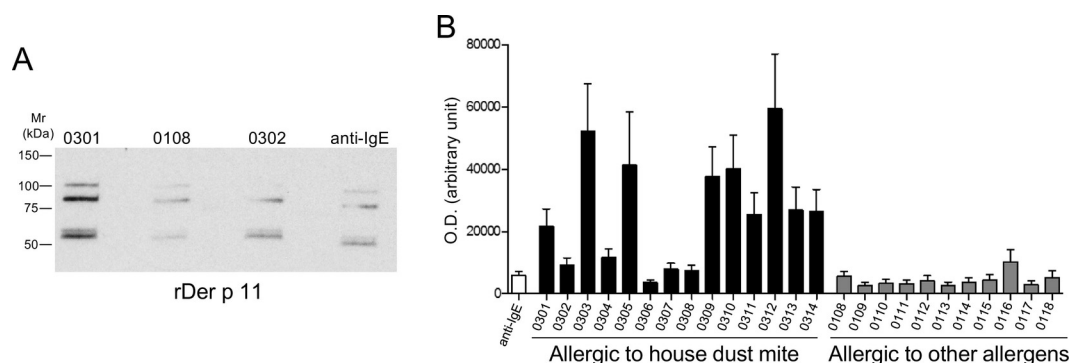


Fig. 3. Reactivity of patient 0301 serum to Der p 11 protein allergen. (A) Recombinant Der p 11 allergen (1.5 µg) was run on SDS-PAGE and probed by western blot with sera 0301, 0302, 0108 and with the anti-human IgE secondary antibody. Reactivity against full Der p 11 molecule (100 kDa) and its degradation products was considered. (B) Semi-quantitative evaluation of western blot IgE optical density (arbitrary units, a.u.) reactivity against rDer p 11 of a cohort of allergic patients sera including both, patients allergic to house dust mite (black bars) and patients allergic to other allergens (grey bars). Three different experiments were performed (n = 3, technical replicates) using n = 14 and n = 11 different sera for the group of patients positive or negative for HDM allergens reactivity, respectively.

technique (dot blot) in a cohort of 100 HDM allergic patients, identified only 2 subjects negative for Der p 1, Der p 2 and Der p 10. The latter included one patient with IgE to Der p 11 and Der p 23, one with IgE to Der p 18. Moreover, the possibility that single allergen components within a given allergen source may serve as markers of defined clinical characteristics is well established. In the specific case, we reported that rDer p 11 might be a useful serological marker allergen for the identification of a subgroup of HDM-allergic patients suffering from HDM-associated atopic dermatitis [14].

Here, we report that Der f 11/Der p 11 was the only detectable sensitizing allergen in a mite allergic child, with a past medical history of atopic dermatitis, in the absence of IgE to the set of the allergen we tested (group 1, 2, 10, 14 and 23 allergens from *D. pteronyssinus* and *farinae* measured by standard ImmunoCAP and ImmunoCAP ISAC assays and/or by western blot). The first group 11 mite allergen was previously identified in *Dermatophagoides pteronyssinus* [14]. Here, for the first time IgE reactivity was detected to group 11 allergen in *D. farinae*. Group 11 allergens belong to a family of proteins known as paramyosins, i.e., muscle associated proteins identified in invertebrates [23]. Paramyosin is a highly immunogenic, muscle-associated alpha-helical protein with, high-molecular-weight (~100 kDa) expressed by invertebrates which had been selected in the attempt to develop a vaccine against parasites (i.e., Schistosomes) [24]. In agreement with high immunogenicity, several reports have suggested that group 11 proteins may also be important allergens [21,25–28]. More recently

[14] the group 11 allergen from *Dermatophagoides pteronyssinus* (Der p 11) was expressed in *Escherichia coli*, purified and biochemically characterized. IgE reactivity to Der p 11 was studied in a group of mite allergic patients stratified into patients suffering from only respiratory forms of HDM allergy and patients with HDM-associated AD [14].

Additional findings about paramyosin stem from the consideration that another allergen associated with mite muscles, i.e., tropomyosin (Der p 10), was more frequently recognized by AD patients than by patients with respiratory allergy to HDM [14].

Our report is in line with the notion that IgE to group 11 mite allergen may co-segregate with diagnosis of atopic dermatitis, at least in the past medical history, also in Italian patients. Notably, the Italian patients who were analysed for IgE reactivity by Banerjee et al. did not include subjects with atopic dermatitis at the time of bleeding, but no information was available about their past medical history. In the small cohort of patients sensitized to inhalant allergens we tested, 36% scored positive for rDer p 11 IgE reactivity, which increased to 64% if only HDM sensitized patients were considered. These proportions are remarkably higher than previously reported (7%) in a group of 67 Italian patients [14], whereas the reactivity to Der p 11 was 55% and 67%, respectively for German and Austrian HDM allergic patients with atopic dermatitis. This discrepancy may depend on several reasons, including the use of different semi-quantitative techniques (WB versus dot blot) not technically validated and not available for widespread clinical use. Another explanation may be the small sample size, which might have selected, by chance, a population enriched for subjects with atopic dermatitis. Moreover, we have no information on the diagnosis of atopic dermatitis in our patients' cohort. Interestingly, Posa et al. [9] suggested that in most HDM positive patients a time-dependent evolution of the reactivity from the most common (e.g. group 1, 2 and 23) toward minor allergens like Der p 11 might occur. In this scenario, regardless the presence of atopic dermatitis, our patients, who are group 1 and 2 positive, could have spread IgE reactivity to group 11 allergens. The high reactivity against paramyosin found in these patients might reflect the HDM specific higher IgE titer showed in comparison to the 0301 patient, which in turn may reflect a longer immunological experience with HDM sensitization [9]. Notably, no reactivity to Der p 23 was detectable in this patient, highlighting the peculiarity of his pattern of sensitization to HDM.

Interestingly, we also observed IgE reactivity of patient 0301 to a group 1 native allergen (Der f 1) which did not correlate with data from ISAC and ImmunoCAP. Group I allergens are cysteine proteases with pro-inflammatory properties [29] eliciting IgE responses in 80% of mite allergic subjects. This observation is in agreement with Becker et al. [4], who similarly observed IgE reactivity to dot-blotted native Der p 1 and high-molecular weight components (>100 kDa) in a group of sera negative for IgE to recombinant Der p 1. This result is compatible with the possibility that the fine specificity of IgE to group 1

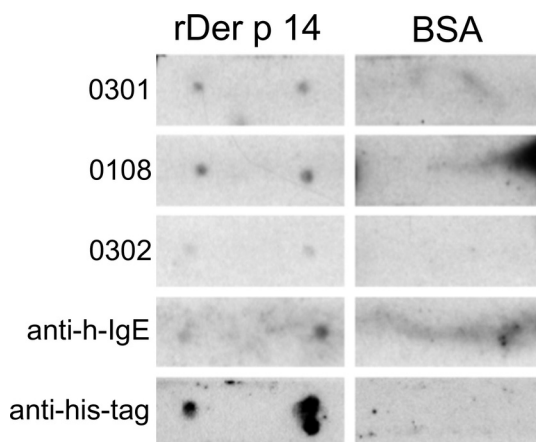


Fig. 4. Reactivity against Der p 14. His-tag recombinant Der p 14 allergen or bovine serum albumine (BSA) were spotted (1.5 µg) in duplicate on nitrocellulose membrane and probed by dot blot for IgE reactivity of sera from patient 0301, 0302, and 0108; anti-human IgE and anti-his-tag secondary antibodies were used as negative and positive control respectively.

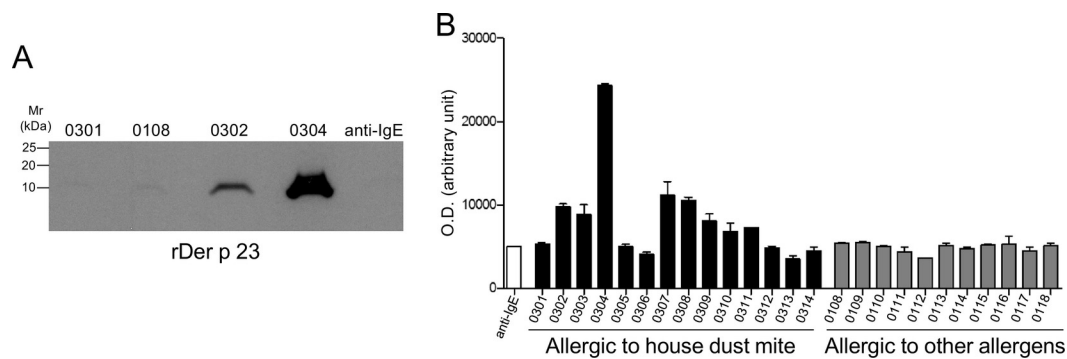


Fig. 5. Reactivity of patient 0301 serum to Der p 23 protein allergen. (A) Recombinant Der p 23 allergen (1.5 µg) was run on SDS-PAGE and probed by western blot with sera 0301, 0302, 0304, 0108 and with the anti-human IgE secondary antibody. Reactivity against Der p 23 molecule (about 9 kDa) was considered. (B) Semi-quantitative evaluation of western blot IgE optical density (arbitrary units, a.u.) reactivity against rDer p 23 of a cohort of allergic patients sera including both, patients allergic to house dust mite (black bars) and patients allergic to other allergens (grey bars). Three different experiments were performed (n = 3, technical replicates).

allergen in some patients can be susceptible to be blunted by such factors as subtle conformational differences, lack of glycosylation and/or dimerization/aggregation. Indeed, the reactivity of patient 0301 to Der f 1 was directed against multimers/aggregates but not against the monomer of about 25 kDa, which is, on the contrary, strongly recognized by the patients 0302, known to be reactive also with the rDer f 1 in ISAC test (Table 2). Alternatively, the reactivity toward macro-aggregates could reflect the propensity of background reactivity of the aggregates as suggested by reactivity of the secondary antibody. Further studies are needed to evaluate IgE reactivity to aggregates of group I allergen components.

The reactivity of patients 0301 IgE against native Der f 14 which was observed at 2DE analysis was not confirmed using the recombinant analogue Der p 14 molecule. Further study is needed to clarify this issue.

In conclusion, our data add up to the notion that in some patients Der p 11 can be an important allergen for mite allergy that should be included in component resolved diagnostic tools.

Conflict of interest disclosure

The authors have no conflict of interest to declare.

Transparency document

The Transparency document associated with this article can be found, in online version.

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