Original Article

Allergenic cross-reactivity between *Blomia tropicalis* and *Blomia kulagini* (*Acari: Echymiopodidae*) extracts from optimized mite cultures

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Abstract. *Background:* At present, data about the cross-reactivity of *Blomia* spp. comes from studies made among different genera of mites, and no results have been published involving different species of the genus *Blomia*.
Objective: The aim of this study was to find out the level of cross-reactivity between the two main species of *Blomia* causing allergy, and its implication in the diagnosis of *Blomia* sensitization.
Methods: Using extracts from optimal growth phases of *Blomia kulagini* (Zakhvatkin, 1936) and *Blomia tropicalis* (van Bronswijk, Cock and Oshima, 1973) as allergenic material, the allergenic cross-reactivity between both house dust mites was evaluated by means of cutaneous tests, specific IgE values, ImmunoCAP-inhibition and SDS-PAGE- IgE-immunoblotting-inhibition.
Results: The results demonstrated that IgE-binding components belonging to both species are very similar from the immunological point of view, showing high correlations between both species when using cutaneous tests (R^2=0.915) or specific IgE (R^2=0.980). ImmunoCAP-inhibition and SDS-PAGE-IgE-Immunoblotting-inhibition probed with human sera, showed a total inhibition of specific IgE reactions by the heterologous antigens.
Conclusion: The results obtained strongly suggest the great resemblance between the allergenic composition of both species.

Key words: *Blomia tropicalis, Blomia kulagini*, mite allergens, cross-reactivity.

Introduction

In the last few years, studies about mite sensitisation prevalence and/or mite fauna in house dust samples from temperate or subtropical regions have shown the increasing importance of some species from *Blomia* as a potential cause of sensitization and allergy [1-5], and suggest that these mites are not located exclusively in tropical or subtropical regions [6,7].

This is supported by the findings of Portús et al. [8], Gómez and Portús [9] , Franjola and Rosinelli [10] and Franz et al. [11], who reported the presence of *Blomia tropicalis* in north-western Spain at sea level, *Blomia kulagini* in north-western Spain at 1200 m of altitude in an alpine climate, and *Blomia tjibodas* in southern Chile and in Germany, respectively.

Several reports on the dual sensitization caused by *Blomia* and *Dermatophagoides* have been published [12-14], and extensive studies have been conducted to evaluate the cross-reactivity of different recombinant allergens of both species [4,5, 12-15, www.allergen.org]. Simpson et al. [5] suggest that sensitization to *B. tropicalis* in the UK, where this mite does not form part of the acarofauna, is caused by IgE-mediated cross-reactivity between allergens produced by *B. tropicalis* and *Dermatophagoides pteronyssinus*.

Despite the different geographical distributions associated to *B. tropicalis* and *Lepidoglyphus destructor*, sensitization to both mite species also has been described [16]. Johansson et al. [17] showed that coexisting sensitization of both Glycyphagoidea could be due to allergenic cross-reactivity between them.

Although mite fauna descriptions of *B. kulagini* and *B. tjibodas* are reported in non-tropical countries, and the emergence of *Blomia* sensitizations in these countries is a fact, extracts from different *Blomia* species not coincident with mite fauna descriptions have been used by some authors as tools for diagnostic purposes [7, 18].

Musken et al. [2] and Franz et al. [11] used by the first time in non-tropical latitudes, extracts from species of *Blomia* found in local dust samples, to measure sensitization to this mite in the population located in the same area.

Despite these data, no studies on immunological cross-reactivity among different species of *Blomia* have been reported to date that we know.

The present study investigates the inter-specific cross-reactivity between optimized *B. tropicalis* and *B. kulagini* allergenic extracts [19], using a patient population from a tropical area, and its implications as a tool for allergy diagnosis.

Material and Methods

Mite Culture

B. tropicalis (Central Science Laboratory, UK) and *B. kulagini* (Department of Parasitology. Faculty of Pharmacy, University of Barcelona, Spain) [9] were cultured on a 1:1 (wt:wt) autoclaved mixture of commercial mouse meal (A.04 Panlab, Barcelona) and dried yeast powder according to Cardona et al [19]. Cultures were prepared by inoculation with 3600 mites in 30 mg of medium and subsequent incubation at 24-26ºC and 75-80% relative humidity maintained by a saturated solution of sodium chloride. Each species was stored in a separated incubator. Each culture was examined regularly and separately to assess mite growth and to rule out possible cross-contamination. Manipulation of cultures was performed in a biological safety cabinet.

At two-week intervals, a small culture sample (50 mg) was used to determine the mite concentration (living and dead mites) by microscope count. After reaching the maximum ratio living mites/dead mites, the cultures were processed to obtain the extracts [19].

Control of purity was performed by periodically checking of cultures (each two weeks), discarding fungal or bacterial contamination and identifying the mite species. The identification of mites was carried out using the keys developed by Colloff and Spieksma [20] and van Bronswijk et al. [21].

Extract Preparation

Extraction from the whole culture was performed by shaking for 6 hours at 4-8ºC in phosphate buffered saline (PBS), pH 7.2 (1 g of raw material/10 ml of buffer). Extract suspension was clarified by centrifugation at 3000 g for 30 min., and the supernatant was filtered through cellulose AP20 filters (Millipore, Bedford, USA). The extracts were thoroughly dialyzed against distilled water at 4ºC for 24 hours, using membranes with a 5000 Da. cut-off point. Dialyzed material was centrifuged at 12,000 g for 30 min. at 4ºC, and the supernatant was freeze-dried [19].

The bicinchoninic acid method revealed protein percentages (mg of protein/100 mg of freeze-dried extract) of 38% and 41%, in the extracts obtained from *B. tropicalis* and *B. kulagini* cultures, respectively.

An extract from the culture medium without mites was also obtained according to the same protocol. The protein percentage of this extract was 19%.

The standardization of extracts was carried out according to the recommendations of the European Academy of Allergy and Clinical Immunology [22].

Skin tests

Twenty-five mite-sensitive patients living in Risaralda (coffee-producing Department, located at the centre of the Andine region in Colombia, approx. 1,500 m altitude) with persistent allergic symptomatic histories and no history of either specific immunotherapy or other sensitizations were recruited.

Selection of patients was performed according to the European Academy of Allergy and Clinical Immunology for extract standardization purposes [22].

Prick puncture was performed according to Dreborg et al. [23] using histamine HCl 10 mg/ml and saline solution as positive and negative controls, respectively.

Measurement of the response was made by digitalization of the weal contours.

A SummaSketch III Graphic Card connected to a computer performed the calculations using the computer-assisted design (CAD) software AutoCAD V.11. [24].

Specific IgE measurements

UniCAP 100 and specific IgE FEIA reagents (Pharmacia Diagnostics, Uppsala, Sweden) were used to measure antibody activity according to the instructions of the manufacturer. Results were expressed in kU/L.

Extracts from *B. tropicalis* and from *B. kulagini* were bound to the solid-phases by coupling 50 ml of biotinylated protein solution to streptavidin ImmunoCAPS (Pharmacia Diagnostics, Uppsala,

Sweden) for 30 min using the UniCAP 100 (Pharmacia Diagnostics, Uppsala, Sweden). *Blomia* extracts (at 0.4 mg/ml protein concentration) were biotinylated using the Biotin Labeling Kit (Roche Diagnostics, Germany) according to the instructions of the manufacturer.

Human Sera

Sera from the above mentioned 25 mite-sensitive patients were obtained. All sera were screened to determine the specific IgE levels against *B. tropicalis* and *B. kulagini* allergens, obtaining titers ≥0.8 kU/L (≥RAST class 2). All patients showed concentrations of specific IgE to *Blomia* higher than specific IgE to other mites (*D. pteronyssinus, L. destructor, Acarus siro and Tyrophagus putrescientiae*). A serum pool was produced using aliquots of each individual serum, and destined for use in the immunoblotting and ImmunoCAP-inhibition methods. Control sera were obtained from non-atopic individuals. Informed consent was obtained in all cases.

ImmunoCAP-inhibition

This method was performed according to the original procedure for RAST-inhibition, described by Yman et al. [25], and modified for the purposes of ImmunoCAP. Briefly, 50 µl of pooled sera from patients sensitive to *Blomia* plus 50 µl of 10-fold dilutions (10-0.001 mg/ml) of allergenic extracts (inhibitors) were added to the solid-phase allergen. Solid phase was obtained by coupling mite extracts to streptavidin ImmunoCAPS as described above.

Inhibition curves were obtained by plotting the percentages of binding of specific antibodies to solid phase versus the logarithm of extract concentration (mg/ml) of the added inhibitor.

A sample of sera plus 50 µl of buffer without inhibitor was used as 100% binding.

The extract concentration able to produce 50% binding was defined as Ag_{50} [26].

SDS-PAGE Immunoblotting-inhibition

Protein separation was performed by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE) using polyacrylamide concentrations of 12.5% and 4% for separating and stacking gels, according to Laemmli [27] as modified by Eraso et al. [28]. An amount of 40 µg of protein, estimated by Bradford's method, was applied per lane.

Separated protein bands were electrophoretically transferred to polyvinyl difluoride membranes (PVDF) (Immobilon-P, Millipore, Bedford, MA, USA) using a horizontal semi-dry transference system [29].

Undiluted pooled sera were incubated for one hour, at room temperature, with equal volumes of *Blomia* extracts or BSA to 100 µg/ml protein (estimated by Bradford's method). Mixtures were centrifuged and supernatants were incubated with *Blomia*-blotted PVDF strips [30].

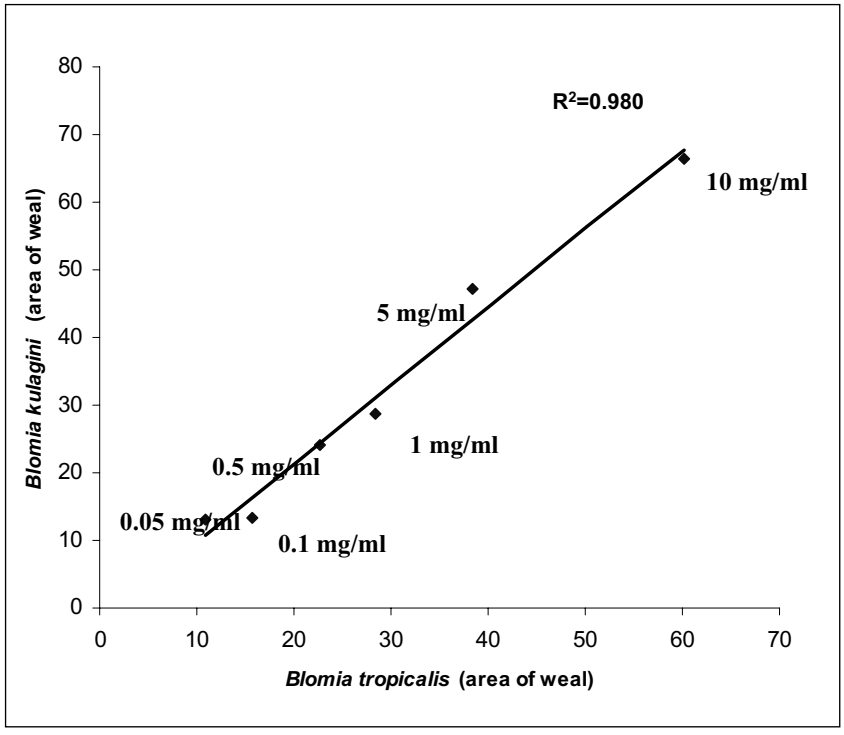

Figure 1. Linear regression between cutaneous responses to *Blomia tropicalis* (Bt) and *Blomia kulagini* (Bk) in the patient population (n=25). The curve was plotted using the mean values of the skin responses (wheal areas in mm^2) to different *Blomia kulagini* and *Blomia tropicalis* extract concentrations (0.05 to 10 mg/ml). Each patient was tested for both species. Correlation analysis yielded a coefficient r=0.990.
Arithmetic mean and standard error of wheal areas produced by the histamine HC1 10 mg/ml, was 28.9 ± 0.52.
Standard errors (σ_M) calculated for each species in each concentration are expressed below: $\sigma_{M\ Bt}$ (0.05 mg/ml) ± 0.11; $\sigma_{M\,Bk}$ (0.05 mg/ml) ± 0.15; $\sigma_{M\,Bt}$ (0.1 mg/ml) ± 0.20; $\sigma_{M\,Bk}$ (0.1 mg/ml) ± 0.26; $\sigma_{M\,Bt}$ (0.5 mg/ml) ± 0.48; $\sigma_{M\,Bk}$ (0.5 mg/ml) ± 0.41; $\sigma_{M\,Bt}$ (1.0 mg/ml) ± 0.66; $\sigma_{M\,Bk}$ (1.0 mg/ml) ± 0.53; $\sigma_{M\,Bt}$ (5.0 mg/ml) ± 1.30; $\sigma_{M\,Bk}$ (5.0 mg/ml) ± 1.48; $\sigma_{M\,Bt}$ (10.0 mg/ml) ± 2.28. $\sigma_{M\,Bk}$ (10.0 mg/ml) ± 3.05.

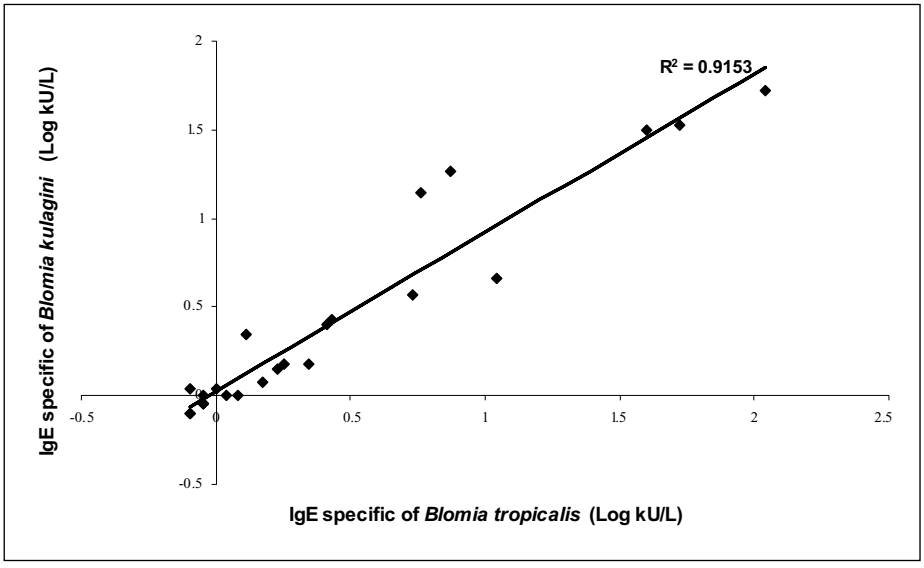

Figure 2. Linear regression between IgE specific of *Blomia tropicalis* and *Blomia kulagini* in the patient population (n=25). Correlation analysis yielded a coefficient r=0.957.

Detection of immune complex was performed according to Shen et al. [31], with modifications during the developing step involving the use of chemiluminescent reagents (ECL+, Western Blotting Detection Reagents, Amersham Biotech).

Protein and allergen patterns were analyzed by GEL COMPAR 4.0 (Applied Maths, Kortijk, Belgium).

Results

Cutaneous response

Figure 1 shows the correlation between cutaneous responses to both *Blomia* extracts, tested in the selected patients. The curve was plotted using the mean values of the skin tests (wheal areas in mm^2) at different *B. kulagini* and *B. tropicalis* extract concentrations. Coefficient calculation (r=0.990) showed an excellent correlation between the cutaneous responses to both species.

Calculation of one histamine equivalent prick revealed by *B. tropicalis* and *B. kulagini* extracts showed values of 0.95 mg/ml and 1.05 mg/ml respectively.

Specific IgE

The specific IgE values for the sera expressed as arithmetic mean ± standard error, were 7.56 ±2.7 for *B. kulagini* and 10.61 ±4.9 for *B. tropicalis*. The comparison of means corresponding to both data populations (not normal distributions, different σ^2) did not show significant differences (Z=0.024, for α=0.01).

Figure 2 shows the correlation between specific IgE values for *B. tropicalis* and *B. kulagini* in the studied population. Coefficient calculation (r=0.957) showed an excellent correlation between the IgE immune responses to both species.

No specific IgE-positive results were shown in the non-allergic control subjects.

ImmunoCAP-inhibition

Table1 shows the ImmunoCAP-inhibition results between both species when *B. tropicalis* and *B. kulagini* were used in solid phase, respectively. The comparison between the homologous systems (intra-specific cross-reactivity, by using the same species in solid phase and

Table 1. Results of ImmunoCAP inhibition between *Blomia tropicalis* and *Blomia Kulagini*.

Solid phase	Inhibitor	r	Slope	Ag_{50} (mg/ml)	% Inhibition
B. kulagini	*B. kulagini*	0.95	23.07	0.070	100
B. kulagini	*B. tropicalis*	0.96	22.31	0.150	100
B. tropicalis	*B. tropicalis*	0.99	8.85	0.008	100
B. tropicalis	*B. kulagini*	0.98	10.76	0.018	100

as inhibitor) and the heterologous systems (inter-specific cross-reactivity) yielded very similar values for both, with theoretical inhibition percentages of 100% in all cases. Also, similar slope values were shown for the homologous and heterologous systems, indicating a similar epitopic variability over the allergenic composition of both species.

Ag50 values obtained for *B. tropicalis* inhibition system were lower than those obtained for *B. kulagini*. These data indicate that the avidity of the specific IgE anti-*B. tropicalis*, was higher than that of the specific IgE anti- *B. kulagini.*

SDS-PAGE IgE-Immunoblotting-inhibition

Figure 3 shows the immunoblotting-inhibition results between the two species. It can be observed that inhibition of IgE immune-reactions by the heterologous allergens was complete, with the exception of two components in the 65-90 kDa range that also appeared in immunoblotting of culture media extracts.

Discussion

In some cases, the species of *Blomia* used to reveal sensitization to this group of mites have not been supported by the species present in the mite-fauna of the related environment [3,7,17,18,32,33].

As initially commented in this study, the reported data about the cross-reactivity of *Blomia* spp. comes from studies made among different genera of mites, and no results have been published involving different species of the genus *Blomia*.

Most studies analyze cross-reactivity between *Dermatophagoides* spp. and *Blomia* spp., using crude extracts [5,7,32-34], or individualized recombinant allergens [4, 5, 12-15, www.allergen.org]. So, Arlian et al. [6] reported limited to moderate cross-reactivity between these two species, and Arruda et al. [15] have in turn demonstrated that 64% of IgE antibodies react species-specifically with *B. tropicalis*, while 36% are cross-reactive with *D. pteronyssinus*.

Simpson et al. [5] suggest that allergens other than those belonging to group 5 are responsible for the partial cross-reactivity between *Blomia* and other mite species, and Thomas et al. [4] indicated that tropomyosin (Blot 10) and paramyosin (Blot 11) could be the main allergens involved in this cross-reactivity phenomenon.

Dual sensitization to *B. tropicalis* and *L. destructor*, both belong to the Glycyphagoidea, has also been shown [16]. Johansson et al. [17] have confirmed allergenic cross-reactivity between both species showing that dual sensitization could be due to immunological similarities between allergens from both Glycyphagoidea, including the major allergen Lep d 2 from *L. destructor*.

Puerta et al. [35] also showed a very significant cross-reactivity between *Suidasia medanensis* and *B. tropicalis* using sera from patients living in Colombia.

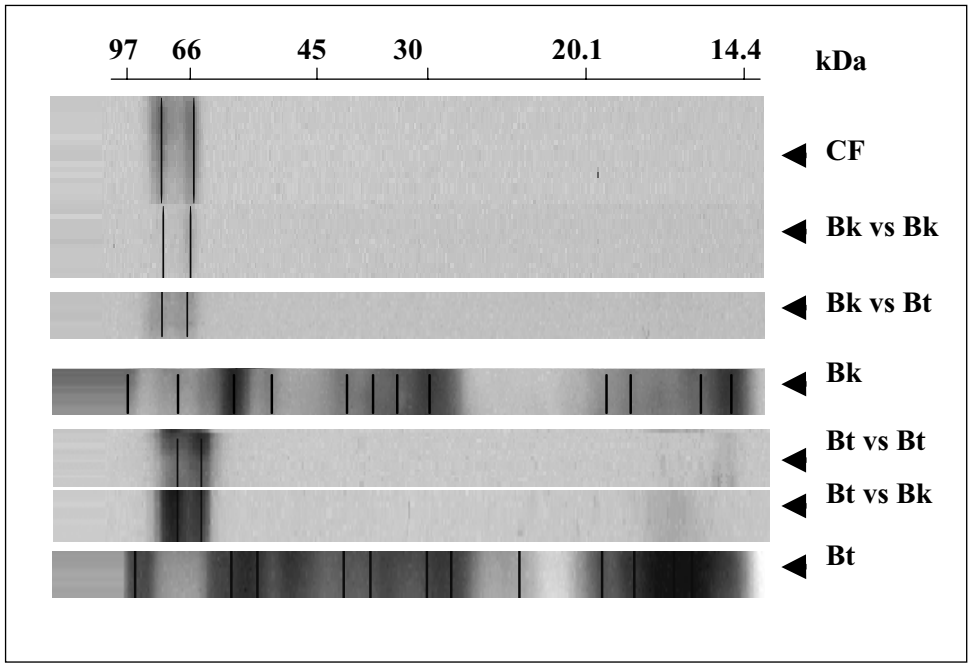

Figure 3. Immunoblotting-inhibition. Extracts from *Blomia tropicalis* and *Blomia kulagini* obtained from cultures showing maximum ratio living mites/death mites during the exponential growth, and undiluted pooled sera from allergic patients to *Blomia*, were used.
CF: Immunoblotting of extract from media used to culture the mites.
Bk: Immunoblotting of *Blomia kulagini.*
Bt: Immunoblotting of *Blomia tropicalis.*
Bk vs Bk: Immunoblotting of *Blomia kulagini* inhibited with *Blomia kulagini.*
Bk vs Bt: Immunoblotting of *Blomia kulagini* inhibited with *Blomia tropicalis.*
Bt vs Bt: Immunoblotting of *Blomia tropicalis* inhibited with *Blomia tropicalis.*
Bt vs Bk: Immunoblotting of *Blomia tropicalis* inhibited with *Blomia kulagini.*

Thus it could be concluded that *Blomia* have significant cross-reactivity with members of superfamilies other than Glycyphagoidea: Acaroidea and Pyroglyphoidea, and also with Lepidoglyphus belonging to Glyciphagoidea. However, no reports studying the cross-reactivity between different species of *Blomia* (Echimyopodidae: Glyciphagoidea) have been described up to now.

Studies of interspecific cross-reactivity in species from the genus *Blomia* could contribute to decide whether it is mandatory to diagnose with extracts from species present in the local acarofauna, or whether it is possible to use other related species despite their absence in the local environment.

Eraso et al. [24], Cardona et al. [19], and Martínez et al. [36] showed that the growth phase of mite cultures is critical to perform diagnostic tests and studies of allergenic cross-reactivity among species belonging to the same genus. They indicate that the extracts obtained from cultures during the growth phase where the ratio living mites/dead mites was maximum, express the highest allergenic variability and potency.

In this context, the ImmunoCAP-inhibition curves obtained in our study were very similar, indicating that the epitope populations contained in both species are practically identical. According to Martinez et al. [26], the difference observed in Ag_{50} values could suggest that the antibody population contained in pooled sera from the selected patients shows that the avidity of IgE anti-*B. kulagini* is higher than that of the IgE anti- *B. tropicalis.* It could also suggest that relative concentration of *Blomia* antigens (number of antigens by unit of protein content) is higher in the extracts from *B. kulagini.*

Theoretical inhibition percentages between homologous and heterologous systems were 100%, thus reinforcing the antigenic similarity of the two species. Martínez et al. [36] in a similar study involving house dust mites *D. pteronyssinus* and *Dermatophagoides farinae* also found high inhibition percentages, even though the maximum theoretical inhibition percentage reached values about 85%.

SDS-PAGE IgE-immunoblotting developed a high intensity in the 14-20 kDa and >30 kDa regions of *B. tropicalis*, but less pronounced in *B. kulagini*. These regions correspond to a range where major Blo t allergens are found (Blo t 5: 14.5 kDa, Blo t 10: 37 kDa). These results together with those reported by Cardona et al. [19], who recommend the inclusion of Blo t 5 major allergen in the group 5 of *Blomia* spp, suggest the direct implication of *Blomia* major allergens in the cross-reactions of both species.

SDS-PAGE IgE-immunoblotting revealed the complete disappearance of IgE-binding components when inhibition was performed with extracts from both species. Only two components in the 65-90 kDa range were not inhibited. The same components were revealed by immunoblotting when extracts from culture media without mites were tested as negative controls, thus supporting the non-specificity of these two bands.

Quantitative and qualitative inhibition experiments clearly showed the antigenic identity of both species, suggesting the possibility of indiscriminately using any species as immunodiagnostic tool. The similarity of the allergenic potency (histamine equivalent prick) shown by the extract from both *Blomia* spp. and the excellent correlation obtained when extracts from both species were used in cutaneous and in "in vitro" tests (ImmunoCAP) support this idea.

Because theoretical inhibition percentages between two different species were 100%, it is possible to think that some experimental artifact could happen. In accordance with García-Robaina et al. [33] and Martinez et al. [36], optimal quality mite allergen extracts and the choice of sera are main parameters to take into account to design cross-reactivity studies by means of EIA-inhibition methods. Regarding this subject and taking into account that the species described in Colombia as predominant house dust mites, are *D. pteronyssinus* and *B. tropicalis* [37], it is possible to affirm that primary sensitisation occurred through *B. tropicalis* exposure, and that the results obtained in this study are not affected by the serum used. On the other hand the taxonomical criteria used by Colloff and Spieksma [20] and van Bronswijk et al. [21] to identify and to separate different species of *Blomia* are mainly supported by a few little morphological differences in *vi*, *ve* or *vg* setae, genital plates or lateral notches and consequently, the approach to the taxonomical differentiation between *B. tropicalis* and *B. kulagini* seems to be very scarce.

The morphological similarities between both mites, and the resulting limitations for the taxonomical identification of *Blomia* species together with high antigenic profiles homology shown in this work, suggest the need to deepen into the taxonomical separation of both species.

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