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Culture of *Blomia tropicalis* and IgE Immunoblot Characterization of Its Allergenicity

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SUMMARY ***Blomia tropicalis*** **is an important triggering factor for allergic diseases such as asthma, rhinitis and atopic dermatitis in tropical and subtropical regions, which climate favours the growth of this species. Our previous mite fauna study revealed that *Blomia tropicalis* is the most dominant species present in Singapore house dust. The main objective of this study is to establish a mass culture of *Blomia tropicalis* for further characterization of the antigenic and molecular properties of this mite. Approximately one gram of mites could be obtained for every 300-gram of culture medium by culturing under natural condition with a mean annual temperature of 30°C and a mean relative humidity of 80%, and harvested by modified Tullgren funnel. Allergen characterization by IgE immunoblot analysis with crude mite extracts showed some IgE reactivity differences between *Blomia tropicalis* mite extract from Singapore and Colombia. The possible reasons for these findings are the quality and source of the mite protein extracts used, or selective differences in the population under evaluation. Further, the atopic sera tested showed differences in the pattern and intensity of IgE immunoblot reactivity to crude extracts of *Blomia tropicalis* and *Dermatophagoides pteronyssinus*, the other highly prevalent mite in Singapore. These data support the existence of species-specific allergens. In conclusion, we have been successful in setting up *B. tropicalis* mass cultures and have prepared extracts of high allergenicity.**

The house dust mite is an important causative agent for common allergic disorders such as asthma, atopic dermatitis, rhinitis and rhinoconjunctivitis.[1,2] It is well-established that pyroglyphid mites, *Dermatophagoides pteronyssinus* and *D. farinae* are widespread sources of potent inhalant allergens in many parts of the world,[3-6] but recently, the clinical importance of the non-pyroglyphid mites, mainly *Lepidoglyphus destructor*, *Blomia tropicalis*, *Acarus siro* and *Tyrophagus putrescentiae*, has been increasingly recognized.[5-8] Among these mite species, *B. tropicalis* is the most ubiquitous in tropical and subtropical regions, where the climate offers an ideal growth condition for them.[6,9,10] *B. tropicalis* are commonly found in Asia-Pacific region.[7,11,12,13] A recent study showed that house dusts in Singapore contained various mite species, with *B. tropicalis* the most predominant, making up 62% of the total count.[14] In addition, allergen distribution and skin prick test surveys also showed that *B. tropicalis* is one of the major allergenic components in the Singapore dust.[15,16] However, many studies showed that there was relatively low cross-reactivity between *B. tropicalis* and the other dominant mite, *D. pteronyssinus*,[3,6,7,17,18,19] which is the second most prevalent species in Singapore.[4] Among *B. tropicalis* allergens that have been cloned and reported so far, only Blo t5 allergen shares 43% protein sequence homology and partial IgE cross-reactivity with Der p5.[20] Therefore, it is important to characterize the allergens of *B. tropicalis*, so that it can be included in

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the panel of therapeutic and diagnostic reagents. To date, there is no *B. tropicalis* extract commercially available, making it essential to culture this mite species, in order to study the clinical importance of *B. tropicalis*.

MATERIALS AND METHODS

Human sera

Human serum samples were obtained from allergic volunteers who had positive skin reactions with *B. tropicalis* and *D. pteronyssinus* extracts. A non-atopic serum, skin test negative to both mite extracts, was used as negative control. The sera were stored at -80°C until use.

Mites source

Lyophilized *D. pteronyssinus* mites were purchased from Commonwealth Serum Laboratory (CSL, Melbourne, Australia). *B. tropicalis* mites were grown in our laboratory and the starter cultures were prepared by collecting mites from the house dust samples in Singapore. *B. tropicalis* were identified according to Bronswijk[9] and isolated under a stereomicroscope from the dust samples, further confirmed by conventional light microscopy at 250x magnification, which were used for setting-up a starter culture.

Method of culture

Fine powdered tetramin fish feed was used as a culture medium for culturing *B. tropicalis*. The tetramin flakes were heated at 60°C for 3 hours before being grounded to kill pre-existing mites and insects. It was then sieved through a 125 μm sieve before use. In order to provide a maximum surface area, a thin layer of < 125 μm particles were introduced into Erlenmeyer flasks together with a few flakes that served as shelters and breeding ground for mites. A starter culture from a small bottle was inoculated into a one-litre Erlenmeyer flask which was then covered by two layers of paper towels to allow ventilation, and sealed with masking tape around the opening of the flask to prevent contamination from the other mites and insects. Those cultures were grown under natural environmental conditions with a mean annual temperature of 30°C and a mean relative humidity of 80%.

Harvesting of mites

Harvesting of mites was performed when the culture was approximately 4-weeks old. Culture was observed under stereomicroscope, and randomly picked cultured mites were further confirmed by conventional light microscopy to determine the purity of the mite populations. The mite culture was separated through a series of 500 μm and 125 μm sieves, by using a mechanical sieve shaker, where vibration was applied for about 20 minutes. Mites with sizes greater than 120 μm were transferred to a modified Tullgren, which was built up of 5 layers of gauze on a funnel that attached to a 15ml-Falcon tube (2097). A 60W bulb was applied from a distance of 15 cm from the culture medium for 4 hours. Most of the mites in the medium crawled through the gauze and down into the tube. Mites that remained in the funnel were then swept into the tube using a tiny soft brush after the gauze was removed. The purified mites were stored at -80°C until use. Mites and the powdered medium particles that were finer than 125 μm collected in a receiving pan placed at the bottom of the sieves were used for further subculturing.

Scanning electron microscopy (SEM)

Mites were isolated from the culture and placed directly onto a sample stub with the presence of carbon tape. The mites were observed under scanning electron-microscope (10kV acceleration voltage for beaming) and pictures were taken at specific magnifications.

Preparation of mite crude extract

One gram of frozen or lyophilized mites was homogenized using a pestle and mortar in the presence of liquid nitrogen. Twenty-five milliliters of phosphate-buffered saline (PBS) containing 2 mM phenylmethyl-sufonyl fluoride (PMSF) and 1 mM EDTA was used for protein extraction at 4°C overnight. After centrifugation at 15,000 x *g* for 15 minutes, the supernatant of the extract was dialysed overnight at 4°C against PBS. The protein concentration of extracts was then determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, USA) and then kept at -80°C.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and IgE immunoblot assay

Immunoblots were prepared by separating about 30 μg/well of mite proteins in the crude extract on a 7.5% SDS–PAGE, followed by electroblotting onto nitrocellulose membranes (Hybond-C extra, Amersham Life Science, England). The IgE immunoassay was per-

formed according to a previously described protocol.[21] Briefly, the blots were blocked in PBS containing 0.05% Tween 20 (PBST) and 5% non-fat milk. After washing, each blot was incubated with 600 µl of individual allergic serum diluted 1:2 in blocking solution overnight at 4°C with continuous shaking. The blots were then vigorously washed thrice with PBST, followed by incubating with 2 µg/ml of biotin-anti human IgE (PharMingen, San Diego, CA) at room temperature for an hour. The blots were then washed as before and were subsequently incubated with ExtrAvidin conjugated with peroxidase (Sigma) (1:4,000) for another hour at room temperature. After washing, the signals were developed with ECL-PLUS Western Blotting Detection System Reagent Kit (Amersham Life Science, England) and autoradiography.

RESULTS AND DISCUSSION

B. tropicalis was obtained from dust samples collected mainly from the kitchen of homes. However, the dust samples contained a mixture of mites[14] with *B. tropicalis* being the most predominant species.

According to van Brownswijk *et al.*,[9] Daphnia is a suitable material to culture *B. tropicalis*. However, as shown in this study, fish food can be a satisfactory medium for culturing *B. tropicalis* too. It has been reported that due to microclimatic fluctuation, there are population differences between natural and cultured mites.[22] In order to mimic the natural conditions as closely as possible, our mites cultures were set up in an uncontrolled environment, with ambient temperature ranging from 26-33°C and RH ranging from 64%-96% throughout the year. A culture took 3-4 weeks to reach the highest population density and subsequently declined (Jimenez Silvia, Personal communication). Therefore, harvesting of *B. tropicalis* has to be performed around the peak period. The results indicates that the growth cycle and conditions of *B. tropicalis* differ from that of *D. pteronyssinus* and *D. farinae*, which takes 12 and 8 weeks, respectively, to obtain the highest yield under 25°C and 75% RH.[19,20]

Using our described culture technique, every 300 grams of culture medium yielded an average of about 1 gram (wet weight) *B. tropicalis* mites. It was however noted that the yield of mites from our mite cultures varied from time to time, ranging from 400 mg to 1.6 gram, we are currently evaluating the effect of RH and temperature on culture yields. However, humidity has been reported as one of the key factors that affect the yield of all species of mite, besides the kind of media use and the period of cultivation.[23,25]

In comparison with various isolation methods that have been reported before,[26] the modified Tullgren funnel used for mite harvesting in this study, was easier and more convenient, avoided the use of organic solvent and saturated salt solution. It is known that fine powder tends to form bigger particles under high humidity condition, which ultimately leads to contamination of medium particles in the mites obtained after sieving. This problem was overcome by using a thicker layer of gauze.

B. tropicalis from our cultures were identified and characterized using scanning electron-microscopy. Figure 1A showing the dorsal view of *B. tropicalis* with 230x magnification, while Fig. 1B is the ventral view of a female mite (190x). Our cultured *B. tropicalis* mites have all the features of *B. tropicalis* that described previously,[9] and generally, female mites made up the majority of the cultured mites.

The extraction process yielded approximately 50 mg crude protein from 1 gram (wet weight) of mites. The protein profile of the *B. tropicalis* extract was then evaluated. Fig. 2 is a SDS-PAGE showing the protein profiles of *B. tropicalis* and *D. pteronyssinus*. Lanes 2 and 3 represent the protein profiles of *B. tropicalis* from Singapore (S) and Colombia (C), respectively. The two profiles showed some minor differences that could probably be explained by the fact that there was a different degree of protein degradation in the two extracts. The extract C that was prepared from the lyophilized mites appeared to have more degradation products than the extract S that was prepared from the relatively fresh frozen mites. There were some significant differences between *B. tropicalis* and *D. pteronyssinus* extracts. Interestingly, the amount of proteins with molecular weights in the 14 kDa region was much higher in *B. tropicalis* compared to *D. pteronyssinus*. Incidentally, at least three *B. tropicalis* allergens identified to date are located in this region.[27,28,29]

The allergenicity of these mite extracts was assessed by IgE immunoblot assay (Fig. 3). Selection of sera used were based on dot blot IgE immunoassay, where a serum with higher titer of specific IgE to *B. tropicalis* than *D. pteronyssinus*, and a serum with stronger IgE reactivity to *D. pteronyssinus*

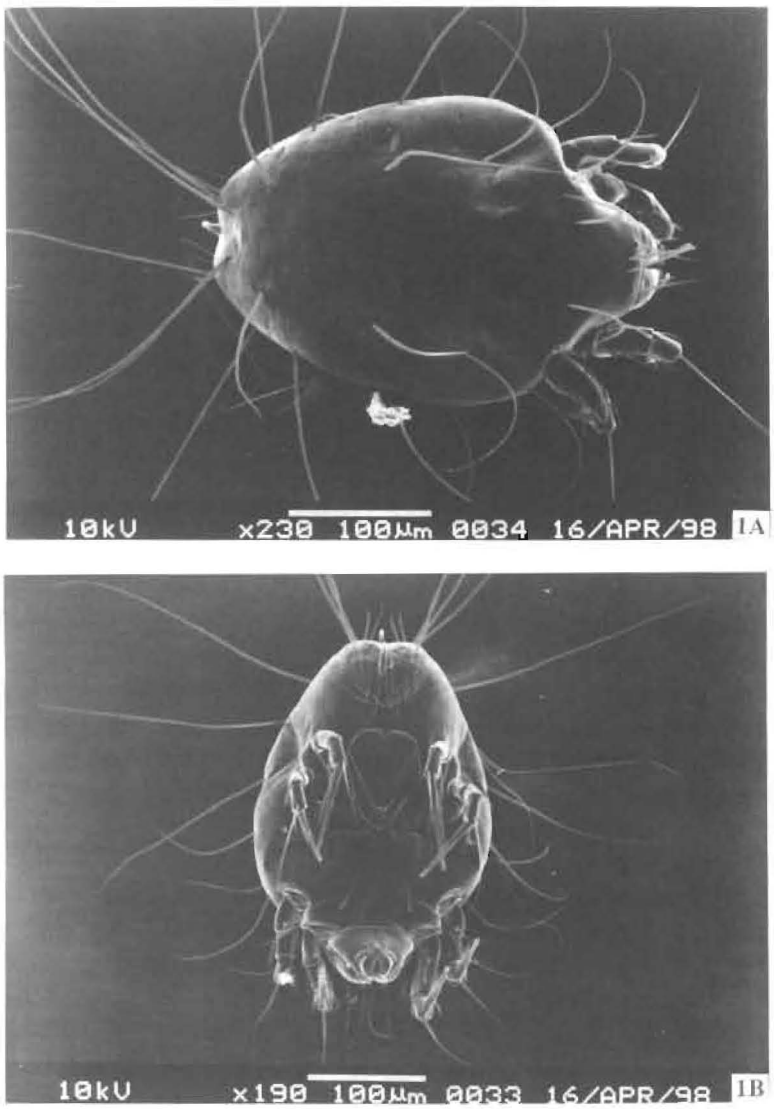

Fig. 1 Scanning electronmicrograph of *Blomia tropicalis*. (A) dorsal view with 230x magnification, and (B) ventral view of a female mite (190x).

than *B. tropicalis*, were used in this immunoblot assay (data not shown). A serum with high *B. tropicalis* specific IgE (Figure 3A) showed strong IgE binding to multiple mite proteins in *B. tropicalis* extract, with molecular weight ranging from approximately 10 kDa to 100 kDa, while serum from an individual with weak *B. tropicalis* IgE reactivity (Fig. 3B) bound to fewer protein bands. The comparison of IgE reactivity profiles of these sera against *B. tropicalis* extracts from Singapore (S) and Columbia (C) showed some interesting differences. As indicated by arrow in Fig. 3A, the serum with high *B. tropicalis*-specific IgE showed stronger binding to proteins in the 31-45 kDa region in extract C compared to extract S. In contrast, the serum with weak *B. tropicalis*-specific IgE (Fig. 3B) showed definite reactivity to a 40 kDa protein band in extract S but not in extract C (indicated by arrow in 3B). Given the fact that the mite extracts were highly susceptible to protein degradation, and the degree of protein degradation is highly dependent on the freshness of the mites for extract preparation,[30] the discrepancies described above may probably reflect the difference in quality of the proteins present in the extracts. However, the possibility that the two extracts may have minor differences in their allergenic components cannot be ruled out, further experiments are required to address such possibility. Genetic differences in the human population (Singapore *vs.* Colombia) eg. HLA profiles, may also exist[31,32] and this may also have an influence on the sensitization pattern to specific allergens. Furthermore, the data obtained indicated that there are greater than 10 allergenic components in the *B. tropicalis* extracts. It is noted that the reactivity of two high molecular weight proteins in both *B. tropicalis and D. pteronyssinus* extracts was due to non-specific binding as indicated by results of the control negative serum, shown in Fig. 3C.

It has been reported that *D. pteronyssinus* is the second most abundant species and allergenic components in Singapore dust,[14] therefore, dual sensitization by *B. tropicalis* and *D. pteronyssinus* mites in Singapore population is expected, and it has been confirmed by *in vitro* IgE-binding data that such dual sensitization is commonly seen in Singapore population.[19] The IgE reactivity to *D. pteronyssinus* extract prepared from commercial lyophilized mites was also evaluated (lanes 3 of Figs. 3A and 3B). Interestingly, the serum

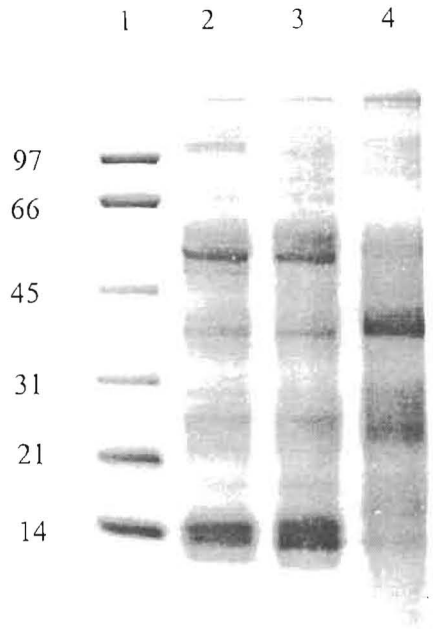

Fig. 2 SDS-PAGE showing the total protein profiles of Singapore (S) and Colombia (C) *Blomia tropicalis* extracts (lanes 2 and 3) and *Dermatophagoides pteronyssinus* extract (lane 4). Lane 1 is protein marker. The gel was stained with Coomassie Brilliant Blue.

with strong reactivity *to B. tropicalis* extract showed low IgE reactivities to *D. pteronyssinus* extract. While most of the IgE reactivity to *B. tropicalis* proteins were targeted at the 14 kDa molecular weight region, there was only a low level of IgE reactivity with *D. pteronyssinus* proteins in the corresponding region. In contrast, most of the IgE in the serum with low *B. tropicalis* reactivity reacted strongly to *D. pteronyssinus* proteins ranging from low to high molecular weights. These data revealed that in Singapore, some subjects are more susceptible to sensitization by *B. tropicalis* than *D. pteronyssinus* mites, and vice versa. Taken together, the data support the existence of species-specific allergens in *B. tropicalis* and *D. pteronyssinus* mites, and therefore justifies the further characterisation of *B. tropicalis* allergens.

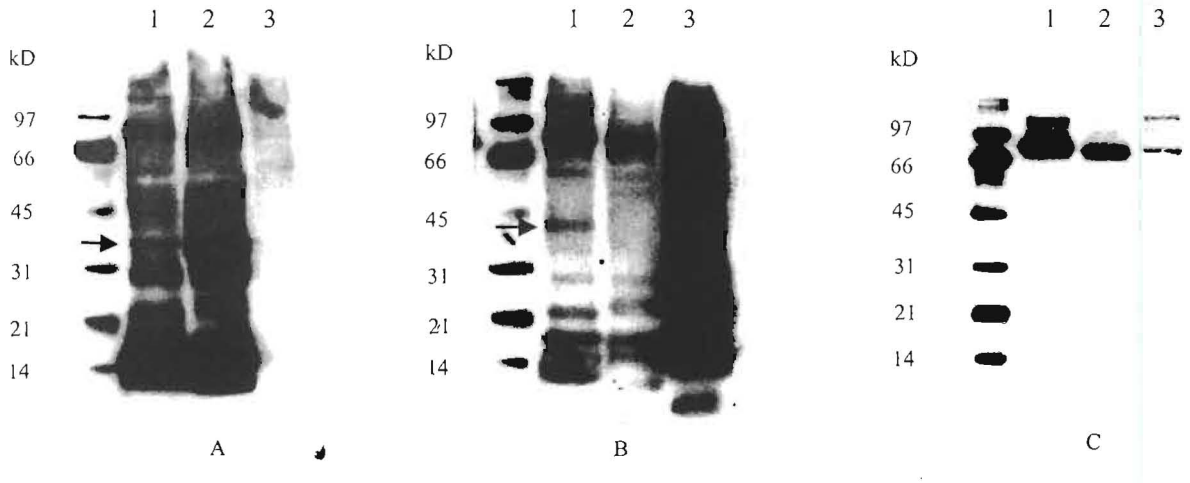

Fig. 3 IgE immunoblotting assay against Singapore *Blomia tropicalis* (lane 1), Colombia *Blomia tropicalis* (lane 2) and *Dermatophagoides pteronyssinus* (lane 3). Panel A was incubated with high *B. tropicalis*-specific IgE serum, panel B low *B. tropicalis*-specific IgE and panel C was negative serum control.

In conclusion, we have successfully set-up *B. tropicalis* mass culture in our laboratory and the extracts prepared from these mites were highly allergenic. This will serve as a source of raw material for allergen identification and characterisation for *B. tropicalis*.

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