

## Original article

# Identification and characterization of a novel allergen from *Blomia tropicalis*: Blo t 21

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**Background:** Allergenic components from *Blomia tropicalis* are important triggers of allergies in the tropics.

**Objective:** We sought to identify and characterize a novel allergen, Blo t 21, from *B tropicalis*.

**Methods:** Blo t 21 was initially identified from an expressed sequence tag database generated from a *B tropicalis* cDNA library. Allergenicity of this antigen was examined by means of skin prick testing, ELISA, and IgE immuno-dot blotting. We evaluated whether Blo t 21 and Blo t 5 were cross-reactive by using IgE inhibition ELISAs.

**Results:** Blo t 21, a 129-amino-acid protein sharing 39% identity with Blo t 5, is a product of a single-copy gene. It has an  $\alpha$ -helical secondary structure and localizes to midgut and hindgut contents of *B tropicalis*, as well as fecal particles. Positive responses to Blo t 21 were shown in 93% (40/43) by means of ELISA and 95% (41/43) by means of skin prick testing when assayed in 43 adult patients with ongoing persistent allergic rhinitis. However, sera of 494 consecutive individuals attending outpatient allergy clinics over 1½ years showed 57.9% (286/494) had positive responses to Blo t 21. Although the majority (>75%) of sensitized individuals were cosensitized to both Blo t 5 and Blo t 21, these 2 allergens had a low-to-moderate degree of cross-reactivity.

**Conclusion:** Blo t 21 is a major allergen in *B tropicalis* that is not highly cross-reactive to Blo t 5, despite sharing some sequence and structural identity.

**Clinical implications:** Blo t 21, representing a new group of allergens, is an important *B tropicalis* allergen. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

**Key words:** Dust mite, *Blomia tropicalis*, allergen, IgE binding, cross-reactivity, immunolocalization, genomic organization

## Abbreviations used

CD: Circular dichroism  
EST: Expressed sequence tagging  
ORF: Open reading frame  
SPT: Skin prick test

*Blomia tropicalis* is a common house dust mite in tropical regions, usually found together with *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, or both and is predominant in Singapore, Malaysia, Brazil, Colombia, and Puerto Rico.<sup>1-5</sup> A high prevalence of *B tropicalis* sensitization occurs and is strongly associated with the presentation of allergic diseases in these countries.<sup>6,7</sup>

Many studies have shown that allergens from *B tropicalis* are species specific, with low-to-moderate cross-reactivity to other dust mite allergens.<sup>8-11</sup> At least 25 IgE-binding protein bands have been identified in *B tropicalis* by means of immunoblotting,<sup>12</sup> of which 10 allergens (Blo t 1, Blo t 3, Blo t 4, Blo t 5, Blo t 6, Blo t 10, Blo t 11, Blo t 12, Blo t 13, and Blo t 19) have been officially named and deposited into the allergen database ([www.allergen.org](http://www.allergen.org)).<sup>13-19</sup> The remaining components have yet to be described.

Most *B tropicalis* allergens identified thus far, however, show sequence homology to *Dermatophagoides* species allergens, except Blo t 12 and Blo t 19. A study in Taiwan showed that Blo t 5 was the most prevalent *B tropicalis* IgE-binding allergen, followed by Blo t 12, Blo t 13, and Blo t 6. Less than 10% of patients responded to Blo t 4 and Blo t 10.<sup>20</sup> Blo t 1 does not seem to share cross-reactive epitopes with Der p 1 or Der f 1<sup>21</sup> and has been reported to be a major allergen,<sup>13,21</sup> but our data seem to suggest otherwise.<sup>8,22</sup>

Our laboratory recently surveyed the transcriptome of *B tropicalis* using an expressed sequence tagging (EST) strategy.<sup>22</sup> EST is a sequence of randomly picked cDNA clones. *B tropicalis* EST information was placed in a database and submitted to the GenBank (CB282036-CB283467). With this database, we were able to predict many more antigens from *B tropicalis* through a bioinformatic approach. These antigens shared sequence homology with allergens from mites, fungi, pollen, food sources, and insects,<sup>22</sup> including a novel antigen that shared a certain degree of sequence similarity to Blo t 5.

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The gene encoding this novel antigen could be a duplicate (paralogous gene) of the Blo t 5 gene in the *B tropicalis* genome. Paralogous genes have a common ancestor in the genome. To verify the allergenicity of this antigen, the response of specific IgE antibodies to this antigen was measured in 494 consecutive individuals attending outpatient allergy clinics by means of immuno-dot blot screening and in 43 allergic patients with persistent rhinitis by means of ELISAs. Skin prick testing was performed in the same group of patients with rhinitis to examine whether this antigen can cross-link IgE on mast cells and trigger the release of proinflammatory mediators, such as histamine, resulting in a wheal response. ELISA inhibition assays were further performed to understand the relationship between this antigen and its homologous allergen Blo t 5, Der p 5, and Lep d 5.

## METHODS

### Serum samples and skin prick tests

Sera were obtained from 43 patients with ongoing persistent allergic rhinitis reporting at least 2 symptoms (nasal obstruction, rhinorrhea, sneezing, and itchy nose) on most days during the past year<sup>23</sup> who were scheduled to start sublingual immunotherapy (the characteristics of the patients are listed in Table E1 in the Online Repository at www.jacionline.org). Their sensitization to both *B tropicalis* and *Dermatophagoides* species, the almost exclusive pattern of persistent allergic rhinitis in Singapore, was confirmed by a positive skin prick test (SPT) reaction. In addition, all had a positive nasal challenge response to crude extracts (0.06 mg/mL) of *B tropicalis*. The challenge procedure was described previously.<sup>24</sup> Sera from 6 healthy adult volunteers with no history of any allergic symptoms and a negative SPT response to common inhalant allergens were obtained as negative controls.

SPTs with the recombinant proteins (0.01 µg/mL in PBS with 50% glycerol) were performed by using a single-prick technique (Solofix, B. Braun, Melsungen, Germany). Negative (PBS with 50% glycerol) and positive (histamine, 10 mg/mL) controls were used for comparison. A wheal response of larger than 3 mm compared with that seen with the negative control after 20 minutes was scored as positive.

In addition, sera from 494 consecutive individuals attending outpatient allergy clinics in the hospital over 1½ years for allergic conditions were screened by using an immuno-dot blot. A total of 262 were children younger than 12 years (mean age, 7.2 years; 59% male; 78% Chinese; 74% had allergic rhinitis, 37% had asthma, 60% had atopic dermatitis, and 14% had clinical manifestation of all 3). The remaining 232 were adults (mean age, 31.2 years; 52% male; 80% Chinese; 100% had allergic rhinitis and 35% had current or childhood asthma). Approval to conduct these studies was obtained from the Institutional Review Boards of the National Healthcare Group, KK Women's and Children's Hospital, and Singapore General Hospital.

### Isolation and genomic organization of Blo t 21 and Blo t 5

Genomic DNA was first isolated by using the method described by Dixit to confirm the presence of these genes.<sup>25</sup> The genes were amplified with the BD Advantage 2 PCR Kit (BD Biosciences). Gene-specific primers were designed according to the 5' and 3' ends of the open reading frames (ORFs) and are listed in Table E2 in the Online Repository (available at www.jacionline.org). They were then cloned into pGEM-T Easy Vector (Promega) and sequenced from both ends.

Southern blotting was then performed to confirm their presence in the genome, as well as to evaluate their relative loci and copy number. Briefly, genomic DNA was completely digested with *Hind*III and *Eco*RV. These enzymes were chosen because the restriction sites were not present in the genes of interest. Probes targeting these genes were generated and labeled with digoxigenin by using the PCR Probe Synthesis Kit, and Southern blotting was performed according to the nonradioactive DIG system (both from Roche Applied Science, Germany).

### Expression, purification, and circular dichroism spectra of the recombinant allergens

The recombinant allergens were expressed and purified as His-tagged proteins in *Escherichia coli* by using the pET expression system (Novagen, Germany). Primers used to amplify the cDNAs are listed in Table E2 in the Online Repository (available at www.jacionline.org). Far-UV circular dichroism (CD) spectra of proteins (at 0.1 mg/mL in 10 mmol/L sodium phosphate buffer, pH 7.4) were measured with a Jasco J810 CD spectropolarimeter from 190 to 260 nm.

### Specific antibody production, Western blotting, and immunolocalization

Polyclonal antibodies against Blo t 5 and Blo t 21 were raised in rabbits by means of subcutaneous injection with 300 µg of recombinant protein with adjuvant and boosted 3 times weekly until high titers were obtained.

Whole mite extracts prepared by using the trichloroacetic acid-acetone method<sup>26</sup> were separated on 15% SDS-PAGE, together with the recombinant proteins in parallel lanes, and transferred onto Hybond nitrocellulose membranes (Amersham Bioscience). Western blotting was performed according to the protocol provided by Amersham Bioscience.

Live mites were fixed, dehydrated, cleared, and embedded in paraffin and then sectioned into slides 8 µm thick to immunolocalize the allergens on mite sections. The sections were deparaffinized, hydrated, and incubated with the respective antibodies according to the Western blotting procedure.

### Immuno-dot blot

One microgram of each recombinant protein was dotted onto nitrocellulose membranes, together with series-diluted IgE standard (National Institute for Biological Standards, United Kingdom) as a positive control, 1 µg of BSA as a negative protein control, and 1 µL of buffer as a control. Membranes were air dried and blocked with PBS–0.1% Tween-20 and incubated with sera (1:1 vol/vol in PBS) at 4°C overnight, followed by 2 hours in anti-human IgE antibodies conjugated with alkaline phosphatase (1:1000 vol/vol in PBS; Sigma). The membranes were then developed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (Promega) for 30 minutes, and spot intensities were measured with imaging software (Microimage v.3.01, Germany). Spot intensities (range, 0–255) were normalized by subtracting the local background, and intensities of greater than 20 (equivalent to 2 SDs above the mean negative sera responses) were considered positive. The interassay and intra-assay concordances were greater than 90% and 95%, respectively, demonstrating good reproducibility of the assay.

### ELISA for quantification of serum-specific IgE

Briefly, 1 µg/mL recombinant proteins were coated onto Maxisorp plates (NUNC, Denmark) and incubated overnight at 4°C. The plates were blocked, washed, and incubated with 50 µL of 1:1 (vol/vol) diluted sera at 4°C overnight. After washing, plates were

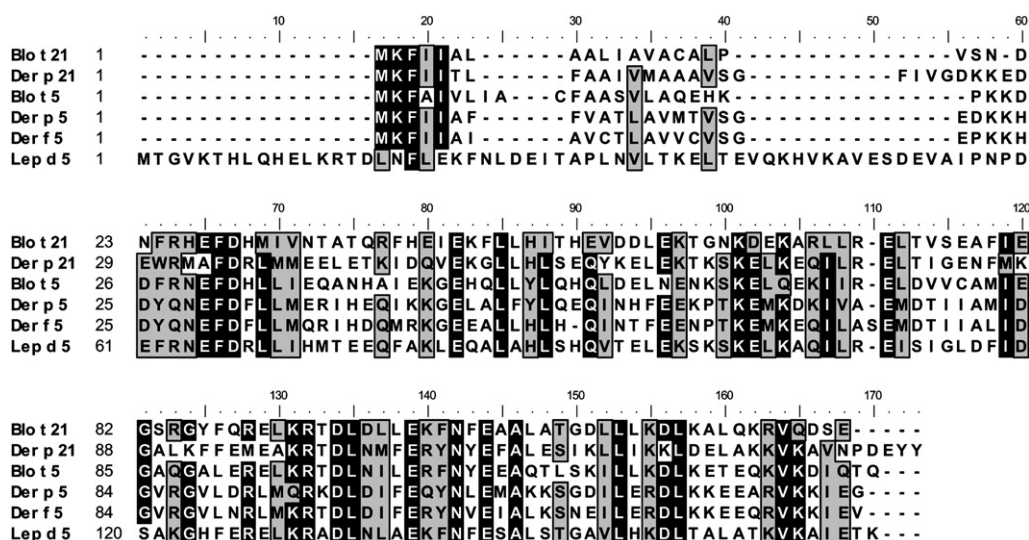


FIG 1. Comparison between Blo t 21 protein sequence with Der p 21 and group 5 allergens. Identical and similar sequences were shaded with black and gray, respectively.

incubated with biotinylated anti-human IgE antibodies and subsequently amplified with avidin–horseradish peroxidase (both from BD Pharmingen). They were then developed with 3,3',5,5'-tetramethylbenzidine (Sigma) and stopped with 1 mol/L hydrochloric acid after 30 minutes. Color intensity was read with a microtiter plate reader at 450 nm. Intensity of greater than 2 SDs of the mean of negative sera responses was considered positive.

### Cross-inhibition of Blo t 5 and Blo t 21 by using competitive ELISA

Inhibition ELISAs were carried out according to a similar protocol described earlier, with the exception that sera were first absorbed with serial 10-fold dilutions of allergens (from 1 ng/mL to 100 µg/mL) overnight. We also performed end point inhibition ELISAs on a larger number of sera where the sera were incubated with 0.1 mg/mL specific allergens overnight before the assay. The percentage of inhibition was calculated by using the following formula:

$$\frac{(OD_{450} \text{ without inhibitor} - OD_{450} \text{ with inhibitor})}{OD_{450} \text{ without inhibitor} - OD_{450} \text{ blank control}} \times 100$$

## RESULTS

### Identification of a novel allergen, Blo t 21, from *B. tropicalis*

We found 3 EST clones generated from a *B. tropicalis* cDNA library<sup>22</sup> to have 40% to 56% amino acid similarities to Blo t 5: 2 full-length clones (CB282881 and CB283036) and 1 truncated clone encoding only the C-terminal region (CB283289). These cDNAs can be assembled into a 605-base-pair contig containing an ORF of 390 base pairs. The ORF encodes 129 amino acids, including a putative 16-amino-acid signal peptide. The calculated molecular weight of the mature protein is 13.2 kd, with a predicted isoelectric point of 5.21 and a predicted α-helix secondary structure (using <http://cubic.bioc.columbia.edu/predictprotein>).

Multiple alignments showed that Blo t 21 shares 30% to 41% amino acid sequence identity with Der p 21 and other group 5 allergens (Fig 1). Pairwise, Blo t 21 shared 41% and 39% amino acid identity with Der p 21 and Blo t 5, respectively. For comparison, Der p 21 shared 31% identity with Der p 5, whereas Blo t 5 and Der p 5 shared 41% identity. However, no clear conserved domain could be observed within and between the group 21 and 5 sequences.

### Genomic organization of the genes encoding Blo t 21 and Blo t 5

Fig E1 in the Online Repository (available at [www.jacionline.org](http://www.jacionline.org)) compares the sequences of these 2 genes, which share 50% nucleotide sequence identity. The Blo t 21 gene has a 56-base-pair intron that interrupts amino acid 18 of the encoded protein. These genes were further examined with Southern blotting. On hybridization, a single band each for Blo t 21 and Blo t 5 was observed in each lane loaded with digested *B. tropicalis* genomic DNA (Fig 2). However, the hybridization patterns for both genes were different. Hence Blo t 21 and Blo t 5 were found to be single-copy genes located at different loci of the *B. tropicalis* genome.

### Secondary structure of Blo t 21 and Blo t 5

Fig 3, A, shows the SDS-PAGE of the purified recombinant proteins, both corresponding to their respective deduced molecular weights: Blo t 5, 14 kd; Blo t 21, 13 kd. The CD profiles showed that these proteins were typical α-helix proteins (Fig 3, B), with 2 maximum negative peaks at 208 and 222 nm and 1 positive peak at 192 nm. The observed secondary structures were similar to those predicted from the amino acid sequences. The CD spectra thus indicated that both Blo t 5 and Blo t 21 shared similar secondary structures and that the recombinant proteins could be properly folded in solution.

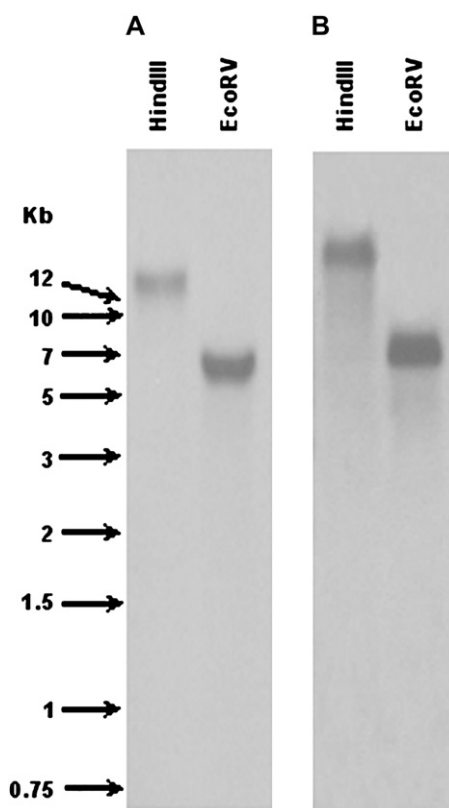


**FIG 2.** Southern blot analysis of Blo t 21 and Blo t 5 genes in the mite genome. **A**, Hybridization with a Blo t 5 probe. **B**, Hybridization with a Blo t 21 probe. In both, genomic DNA was digested with the restriction enzymes *HindIII* and *EcoRV*, respectively.

### Examination of nBlo t 5 and nBlo t 21

Total mite extract was separated on a 15% SDS gel together with the recombinant proteins in parallel lanes (Fig 4, A). In Fig 4, B, Blo t 5 polyclonal antibodies recognized rBlo t 5 but not rBlo t 21. An nBlo t 5 protein at approximately 15 kd was recognized by the antibody. Two other bands (approximately 20 and 28 kd) in the mite extract were also recognized. Mass spectrometric analysis showed that they were similar to Blo t 5, except for a non-specific 25-kd band (denoted with an asterisk) detected in all 3 membranes, including the negative control (Fig 4, D). We also observed that rBlo t 5 exists as a homodimer at 30 kd and a homotrimer at 45 kd.

In contrast, Blo t 21-specific polyclonal antibody reacted strongly to rBlo t 21 but showed a weak cross-reaction to rBlo t 5 (Fig 4, C). Consequently, 2 bands at approximately 15 kd were observed in the lane loaded with crude mite extract. The band that ran slightly higher was identified as Blo t 5 by means of mass spectrometry (data not shown), whereas the lower band was identified as Blo t 21. The nBlo t 21 size matched its predicted molecular weight based on its amino acid sequence. A band that appeared at approximately 20 kd was found to be similar to Blo t 5. Similarly, rBlo t 21 also exists as a homodimer at 30 kd and a homotrimer at 45 kd.

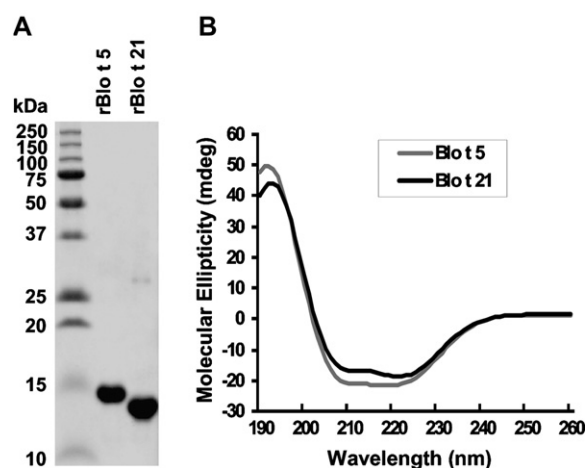


**FIG 3.** Expression of rBlo t 21 and rBlo t 5 allergens and their far UV CD spectra. **A**, Protein profile of rBlo t 21 and rBlo t 5 separated on a 15% SDS-PAGE gel. **B**, Far UV CD spectra of rBlo t 21 and rBlo t 5 proteins in solution.

Sections of mites were immunostained with both antibodies. Anti-Blo t 21 antibody bound strongly to midgut contents and fecal pellets in the hindgut of the sections (Fig 5, A). In parallel, anti-Blo t 5 antibody stained similar positions in the mite sections (Fig 5, B). Anti-Blo t 21 was preabsorbed with Blo t 5 before immunostaining to ensure that the antibodies were not cross-reacting. A preimmune sera control did not stain the mite sections. We have thus shown that Blo t 21 and Blo t 5 co-localize in similar positions and might likely exist in the environment both within mite bodies and in fecal particles.

### Blo t 5 and Blo t 21 sensitization

A total of 40 (93%) of 43 adult patients with allergic rhinitis had specific IgE to Blo t 21, whereas 42 (98%) of 43 had specific IgE to Blo t 5. Patients responding to Blo t 21 also responded to Blo t 5 (Fig 6, A). Among the 494 consecutive individuals attending outpatient allergy clinics, 83.4% (412/494) had sera that were positive to *B. tropicalis*, 57.9% (286/494) had sera that were positive to Blo t 21, and 56.3% (278/494) had sera that were positive to Blo t 5. The majority (>75%, 244/320) responded to both allergens (Fig 6, B). There were no significant differences in the percentage of respondents to Blo t 21 when the individuals were stratified by age group (adults vs children), demographic background (sex or ethnicity) and clinical diagnosis (asthma, allergic rhinitis, and atopic dermatitis), with the exception of children who presented with all 3 clinical manifestations, all showing a positive response to Blo t 21.

SPTs were performed on the same group of patients with allergic rhinitis to further confirm the allergenicity *in vivo*. Two nonatopic volunteers were also evaluated. All patients had a positive response to at least 1 of the allergens, with most ( $n = 41$ ) reacting to both (Fig 6, C). The mean wheal diameter was 5.7 mm for Blo t 5 and 6.2 mm for Blo t 21. Wheal responses against the 2 allergens were,

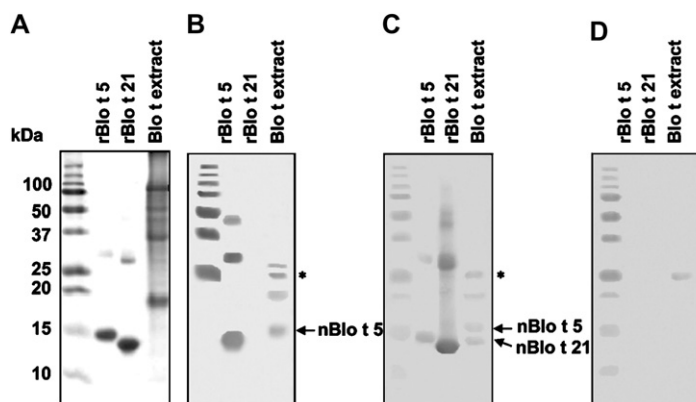


**FIG 4.** Detection of nBlo t 5 and nBlo t 21 allergens in the crude extract. **A**, SDS-PAGE gel image of proteins loaded onto the gel. Immunoblotting images with anti-Blo t 5 (**B**) and anti-Blo t 21 polyclonal antibodies (**C**) and PBS (as a negative control; **D**). A nonspecific band (\*) was detected in all 3 immunoblotting membranes.

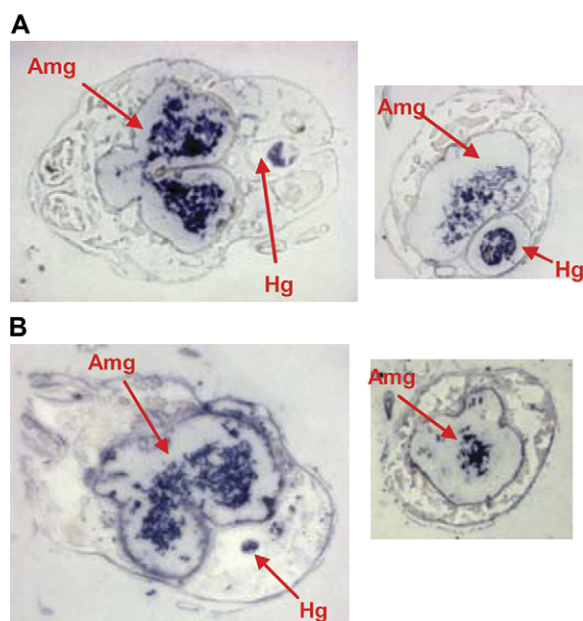


**FIG 5.** Immunostaining of Blo t 21 and Blo t 5 in paraffin-embedded sections of *B tropicalis*. **A**, Probe with anti-Blo t 21 polyclonal antibody in longitudinal and sagittal mite sections. **B**, Probe with anti-Blo t 5 polyclonal antibody. Amg, Anterior midgut; Hg, hindgut.

however, not correlated. In addition, control individuals did not show any wheal-and-flare reactions (not shown).

### IgE cross-reactivity between Blo t 21 and Blo t 5

Cross-reactivity between these allergens was evaluated by means of inhibition ELISAs with 3 sera with high specific IgE titers to both allergens. IgE binding to Blo t 21 could be inhibited by the antigen itself at very low concentrations (0.01  $\mu\text{g/mL}$ ) and achieved full inhibition at 10  $\mu\text{g/mL}$  protein (Fig 7, A). Cross-inhibition by group 5 allergens, however, only occurred after 10  $\mu\text{g/mL}$  in 2 of the 3 sera, whereas no cross-reactive IgE between Blo t 21 and group 5 allergens could be detected in serum V1. In

parallel, on Blo t 5-coated plates, the inhibition curves show that partial cross-reactivity between Blo t 5 and Blo t 21 and other group 5 allergens exists (Fig 7, B). Cross-inhibition with BSA was performed as a control, and no inhibition was observed, even at 100  $\mu\text{g/mL}$  protein (data not shown).

To evaluate further, 10 additional sera with specific IgE to both allergens were used in an end point inhibition experiment using only 100  $\mu\text{g/mL}$  inhibitor (tabulated in Table E3 in the Online Repository at [www.jacionline.org](http://www.jacionline.org)). On average, IgE binding to Blo t 21 was only partially inhibited by group 5 allergens (mean, <30%). In parallel, IgE binding to Blo t 5 was partially inhibited by Blo t 21, Der p 5, and Lep d 5 at a mean of 33%, 51%, and 34%, respectively. Taken together, these experiments indicate that Blo t 21 has low-to-moderate cross-reactivity to group 5 allergens.

## DISCUSSION

We have identified a new allergen, Blo t 21, which is a product of a single-copy gene that shares some similarities to genes encoding group 5 allergens. CD spectra of Blo t 21 and Blo t 5 were similar and typical of  $\alpha$ -helical proteins. These similarities between the primary and secondary structures of group 21 and 5 allergens, as well as their colocalization in mites, seem to suggest that these proteins possibly have related functions. The conservation of amino acid sequence in different proteins originating from the same species is likely due to a prior gene duplication event within the genome of the organism. Duplicated genes (also called paralogs) might, however, change quickly before selective pressure sets in on its new function. Hence duplicated gene products might have modified structures and functions over time while showing some residual resemblance to each other.

We have also isolated group 5 and 21 homologues from other dust mites, such as *Tyrophagus putrescentiae*, *Suidasia medanensis*, and *Aleuroglyphus ovatus* (unpublished data, 2006), whereas Vrtala and colleagues

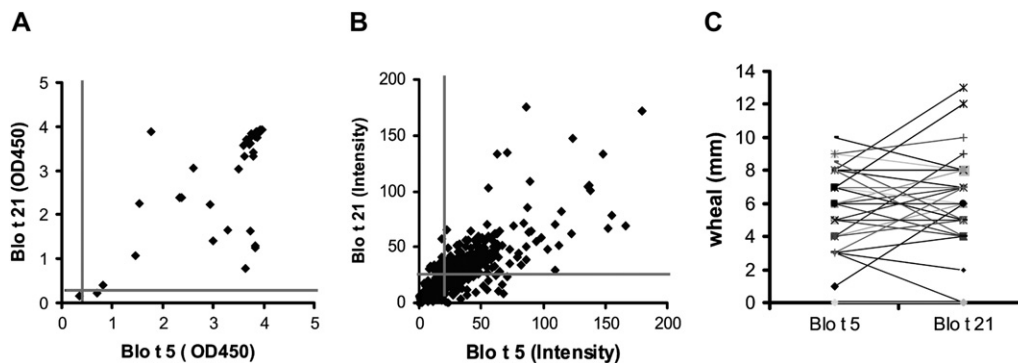


**FIG 6.** Allergenicity of Blo t 21 and Blo t 5. **A**, Specific IgE levels assessed by means of ELISA in sera of 43 patients with allergic rhinitis. **B**, Specific IgE levels assessed by means of dot blot immunoassay in sera of 494 atopic individuals. *Gray lines* indicate the negative cutoff point. **C**, SPT responses among the 43 patients with allergic rhinitis.

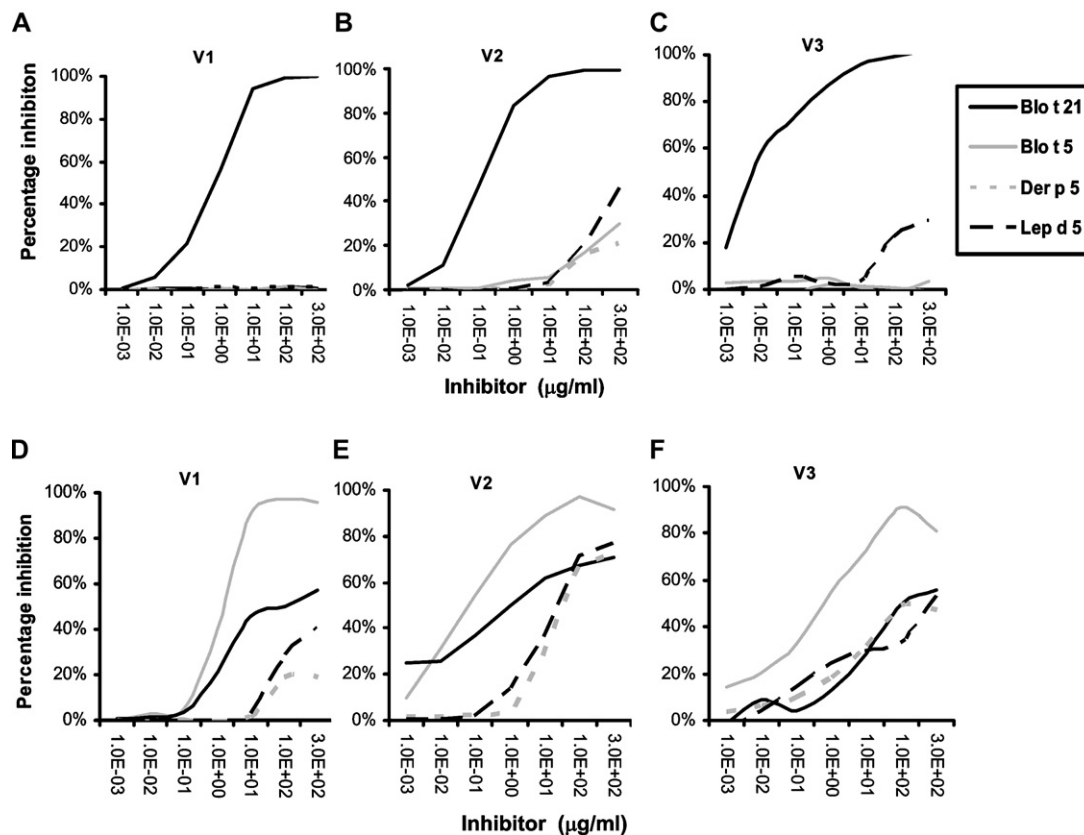


**FIG 7.** Dose-response competitive ELISA assay evaluating the IgE cross-reactivity of Blo t 21 and Blo t 5. Three individual sera were preabsorbed with 10-fold serial dilution of inhibitors and then assayed by using allergen-specific IgE ELISA. **A**, Blo t 21 immobilized on the solid phase of ELISA plates. **B**, Blo t 5 immobilized on the solid phase of ELISA plates.

independently isolated Der p 21 (deposited into the GenBank, ABC73706). The event of paralogy most likely took place before speciation because group 5 and 21 allergen sequences are clustered in different clades when compared phylogenetically. In addition, we observed from our EST database<sup>22</sup> that there is a third paralogous group sharing sequence and structural similarities to both these

allergens and that is capable of binding IgE as well. The gene duplication event resulting in the third paralog, however, might have occurred after speciation because the genes seem to cluster within species (unpublished data).

Many mite proteins around the 15-kd size range are allergenic, including the group 2, 5, 13, and 21 allergens. Because of the lack of genomic information, discovery of

new allergen by using immunoblotting combined with standard molecular techniques can be difficult. Using the EST approach,<sup>22</sup> we have identified paralogs similar to other mite allergens, including the group 2, 7, and 13 allergens.<sup>27-29</sup> This indicated that paralogs are not rare but rather a common phenomenon in the mite genome. It is, however, intriguing to note that paralogous proteins, while sharing similar structures but not necessarily similar epitopes, are also allergenic.

The high percentage of cosensitization to group 21 and 5 allergens observed in this study was consistently seen in other populations (data not shown). These include allergic individuals from Europe, Australia, and South Africa responding to the *Dermatophagoides* group 5 and 21 allergens.<sup>30</sup> We initially reasoned that this might be a result of cross-reactions through the presence of common epitopes. However, inhibition experiments showed that these groups of allergens are not highly cross-reactive to one another and strongly support the idea that group 21 is a distinct group of allergens. They demonstrate low cross-reactivity between species as well, and this is in agreement with the findings of studies conducted elsewhere.<sup>31</sup>

The strong cosensitization profile not ascribed to antigen cross-reactivity could then be due to at least 2 other reasons. First, group 5 and 21 allergens could both have similar abilities to cause sensitization in susceptible individuals and thus be present in similar and correlated levels in the environment. This is likely because our preliminary data show that Blo t 21 and Blo t 5 levels in the environment are relatively similar and correlated to one another. Second, allergen-specific IgE responsiveness has been shown in some cases to be restricted by certain HLAs, and it is possible that immune responses to the 2 antigens are restricted by similar host factors. We have previously shown that HLA-DRB1\*09 is a marker of IgE nonresponsiveness to Blo t 5 among patients from Singapore<sup>32</sup> but not to Der p 5. Whether this is the case for Blo t 21 remains to be evaluated.

We noticed that charged and polar residues on the surface of protein molecules are important for IgE binding, particularly for inhalant allergens.<sup>33</sup> Blo t 21 and Blo t 5 both have more than 40% charged and 20% polar residues throughout the entire sequence. This high level of solvent-accessible residues could allow antibodies to bind more readily to them and be a basis for their allergenicity.

This study clearly showed that sensitization to Blo t 21 is important for *B tropicalis* allergy, although most patients were cosensitized to Blo t 5 at the same time. In tropical regions like Singapore, temperature and humidity are relatively constant and suitable for mite proliferation. It is difficult to completely avoid *B tropicalis* mite. Allergen-specific immunotherapy is thus the only potential specific treatment for IgE-mediated *B tropicalis* allergy. In current clinical practice, aqueous extracts of dust mite were applied for diagnosis and immunotherapy. However, natural allergen extracts consist of complex mixtures of allergenic and nonallergenic components or even endotoxin.<sup>34,35</sup> Recombinant allergens can potentially be used in diagnosis and treatment to enhance the clinical efficacy to

improve the quality and consistency of vaccine. rBlo t 5 and rBlo t 21 used in this study were folded and exhibited IgE-binding reactivity. Although the IgE-binding activity of rBlo t 21 and rBlo t 5 was not compared with that of natural counterparts in this study, it has been reported that rBlo t 5 expressed in *E coli* has comparable IgE binding activity versus nBlo t 5.<sup>36</sup> We believed that rBlo t 21 might also have comparable IgE-binding activity to its natural counterpart like rBlo t 5. Recombinant protein can be produced with high purity. Therefore rBlo t 5 and rBlo t 21 produced in the laboratory can possibly facilitate the substitution of natural allergens in specific diagnosis and immunotherapy. In addition, positive responses to Blo t 21, together with other *B tropicalis* major allergens, might be indicative of more severe atopic disease or concomitant clinical manifestation of all 3 atopic diseases in the tropics.<sup>37</sup> We have observed that significant increases in the number of sensitizations to discrete mite allergenic groups (including the group 21 allergens) in atopic children correlates well with an increasingly complex allergic phenotype (manifestation of all 3 atopic diseases). Whether this can be used as a predictive risk factor for future development of asthma and allergic rhinitis in young children presenting first with atopic dermatitis will require further prospective cohort studies.

In conclusion, we describe here a novel allergen from *B tropicalis*, Blo t 21, that is able to elicit and bind specific IgE in more than 50% of atopic individuals with dust mite allergy. Although Blo t 21 shares some primary and secondary structural similarities to Blo t 5, IgE binding this allergen do not cross-react with Blo t 5. Blo t 21 is thus an important allergen from *B tropicalis* and should be considered in the diagnosis and treatment of dust mite allergy, particularly in tropical regions.

The name Blo t 21 was accepted by the World Health Organization/International Union of Immunological Societies Subcommittee of Allergen Nomenclature. The GenBank accession numbers for the nucleotide sequence encoding Blo t 21 is AY800348. The GenBank accession numbers for 7 Blo t 21 and 9 Blo t 5 genomic organizations variants are DQ788677-DQ788683 and DQ788684-DQ788692, respectively.

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**TABLE E1.** Characteristics of the study patients (n = 43)

|                                    | Descriptive data, n (%) | Measurements, mean ( $\pm$ SD) |
|------------------------------------|-------------------------|--------------------------------|
| Sex                                |                         |                                |
| Male                               | 21 (49%)                |                                |
| Female                             | 22 (51%)                |                                |
| Age (y)                            | 31.5 $\pm$ 9.81         |                                |
| Ethnic representations             |                         |                                |
| Chinese                            | 35                      |                                |
| Malay                              | 3                       |                                |
| Indian                             | 2                       |                                |
| Other                              | 3                       |                                |
| Diagnosis                          |                         |                                |
| Persistent allergic rhinitis       | 43 (100%)               |                                |
| Asthma                             | 9 (21%)                 |                                |
| Childhood asthma                   | 6 (14%)                 |                                |
| Duration of disease (y)            | 14.91 $\pm$ 6.92        |                                |
| Nasal symptom score*               |                         |                                |
| Itchy nose                         |                         | 2.00 $\pm$ 0.87                |
| Sneezing                           |                         | 2.23 $\pm$ 0.72                |
| Rhinorrhea                         |                         | 2.53 $\pm$ 0.67                |
| Nasal obstruction                  |                         | 2.51 $\pm$ 0.70                |
| SPT† (wheal size and % positive)   |                         |                                |
| Der p                              |                         | 8.22 $\pm$ 4.04                |
| Der f                              |                         | 9.05 $\pm$ 3.40                |
| Blo t                              |                         | 8.43 $\pm$ 2.74                |
| Blo t (housemade‡)                 |                         | 7.43 $\pm$ 1.98                |
| IgE test (CAPs; % positive)        |                         |                                |
| Total IgE (>100 IU)                |                         | 81% (35/43)                    |
| (>50 IU)                           |                         | 98% (41/43)                    |
| Der p (>0.5 IU/mL, $\geq$ class 3) |                         | 93% (40/43)                    |
| Der f (>0.5 IU/mL, $\geq$ class 3) |                         | 98% (42/43)                    |
| Blo t (>0.5 IU/mL, $\geq$ class 3) |                         | 95% (41/43)                    |

*Der p*, *Dermatophagoides pteronyssinus*; *Der f*, *Dermatophagoides farinae*;  
*Blo t*, *Blomia tropicalis*.

\*Symptoms are present more than 4 days a week during the past year.

These symptoms were recorded by using a symptom severity scale:

0 = none, no symptom evident; 1 = mild, symptom clearly present but minimal awareness; 2 = moderate, definite awareness of symptom that is bothersome but tolerable; 3 = severe, symptom is hard to tolerate.

†Standard crude allergen extracts from Stallergene.

‡Crude allergen extract made by our own laboratory.

**TABLE E2.** Primers used for genomic DNA amplification and the generation of expression clones

| Primer name | Primer sequence                            | Restriction enzyme | Amplicon             | Vector |
|-------------|--------------------------------------------|--------------------|----------------------|--------|
| Primer 1    | ATGAAATTTATCATCGCATTGGC                    |                    | Blo t 21 genomic DNA | pGEMT  |
| Primer 2    | CTATTCGGAAGCTTGGACTCGC                     |                    | Blo t 21 genomic DNA | pGEMT  |
| Primer 3    | ATGAAGTTCGCCATCGTTCCTTATTGCC               |                    | Blo t 5 genomic DNA  | pGEMT  |
| Primer 4    | TTTATTGGGTTTGAATATCCTTC                    |                    | Blo t 5 genomic DNA  | pGEMT  |
| Primer 5    | GGGAC <b>ATATG</b> CAAGAGCACAAAGCCAAAGAAGG | <i>NdeI</i>        | Blo t 5 cDNA         | pET15  |
| Primer 6    | CGGG <b>ATC</b> CTTATTGGGTTTGAATATCCTTC    | <i>BamHI</i>       | Blo t 5 cDNA         | pET15  |
| Primer 7    | GCGGG <b>ATC</b> CCCTACCAGTCTCTAACGATAAC   | <i>BamHI</i>       | Blo t 21 cDNA        | pETM   |
| Primer 8    | CGGG <b>AAT</b> TCTTATTTCGGAATCTTGGACTCGC  | <i>EcoRI</i>       | Blo t 21 cDNA        | pETM   |
| Primer 9    | GGGAC <b>ATATG</b> GGAAGATAAAAAACATGATT    | <i>NdeI</i>        | Der p 5 cDNA         | pET15  |
| Primer 10   | CGGG <b>ATC</b> CTTAAACTTCAATCTTTTAAACAC   | <i>BamHI</i>       | Der p 5 cDNA         | pET15  |
| Primer 11   | CCCAC <b>ATATG</b> ACCGGTGTCAAGACTCA       | <i>NdeI</i>        | Lep d 5 cDNA         | pET15  |
| Primer 12   | CGGG <b>ATC</b> CTTATTTGGTTTCGATGGCCT      | <i>BamHI</i>       | Lep d 5 cDNA         | pET15  |

Restriction enzyme sites are indicated in boldface.

**TABLE E3.** Quantitative end point cross-inhibition of IgE binding to Blo t 21 and Blo t 5 in sera of 10 patients with allergic rhinitis

| Sera | A. Inhibition of IgE binding to Blo t 21 |         |         |         | B. Inhibition of IgE binding to Blo t 5 |          |         |         |
|------|------------------------------------------|---------|---------|---------|-----------------------------------------|----------|---------|---------|
|      | Blo t 21                                 | Blo t 5 | Der p 5 | Lep d 5 | Blo t 5                                 | Blo t 21 | Der p 5 | Lep d 5 |
| R4   | 80%                                      | 17%     | 16%     | 19%     | 80%                                     | 29%      | 66%     | 54%     |
| R5   | 98%                                      | 11%     | 14%     | 17%     | 95%                                     | 28%      | 65%     | 26%     |
| R7   | 94%                                      | 44%     | 68%     | 53%     | 92%                                     | 32%      | 66%     | 51%     |
| R11  | 87%                                      | 50%     | 23%     | 25%     | 73%                                     | 61%      | 29%     | 34%     |
| R14  | 97%                                      | 53%     | 65%     | 59%     | 90%                                     | 57%      | 74%     | 56%     |
| R18  | 91%                                      | 5%      | 5%      | 6%      | 88%                                     | 19%      | 60%     | 42%     |
| R20  | 97%                                      | 10%     | 16%     | 12%     | 91%                                     | 19%      | 73%     | 49%     |
| R22  | 91%                                      | 2%      | 2%      | 8%      | 75%                                     | 0%       | 23%     | 0%      |
| R23  | 95%                                      | 48%     | 34%     | 31%     | 83%                                     | 78%      | 40%     | 32%     |
| R24  | 96%                                      | 8%      | 14%     | 2%      | 80%                                     | 3%       | 12%     | 0%      |
| Mean | 93%                                      | 25%     | 26%     | 23%     | 85%                                     | 33%      | 51%     | 34%     |

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      10      20      30      40      50      60      70
gBlo t 5  ATGAAGTTCGCCATCGTTCTTATGCGCTGCTTTGCCGCTTGGTTT-----
gBlo t 21 ATGAAATTATCATCGCATTGGCTGCCCTCATCGCAGTGGCATGTGgtaagttggtagctaattattag

      80      90      100      110      120      130      140
gBlo t 5  -----TGGCTCAAGAGCACAGCCAAAGAAGGATGATTCCGA
gBlo t 21 ttgacccaaaaattgaccaacttgatatattagCCCTACCACTCTCTAAC-----GATAACTTCCGT

      150      160      170      180      190      200      210
gBlo t 5  AACGAATTCGATCACTTCTTGATCGAACAGgtaaacagttttttttgaaaggttaaaaaagtaatc
gBlo t 21 CACGAGTTTGACCATATCATCGTCAACACC-----

      220      230      240      250      260      270      280
gBlo t 5  aaagaaactttttagGCAAAACCATGCTATCGAAAGGGAGAACATCAATTCTTTACTTGCAACACCAAC
gBlo t 21 -----GCTACTCAACGATTCATGAGATTGAAAAATTCTTCTGCATATTACTCATGAAG

      290      300      310      320      330      340      350
gBlo t 5  TTGACGAATTGAATGAAAACAAGACCAAGGAATTGCAAGAGAAATCATTGAGAACTTGATGTTGTTTG
gBlo t 21 TTGATGATTTGAAAAAACCGGCACAAAGATGACAAAGCACGACTTCTCCGAGAACTTACCGTTTCTGA

      360      370      380      390      400      410      420
gBlo t 5  CGCCATGATCGAAGGAGCCCAAGGAGCTTTGGAACGTGAATTGAAGCGAACTGATCTTAACATTTTGAA
gBlo t 21 GGCTTTCATCGAAGGATCAAGAGGATTTCCAACGGGAACTCAAGCGAACTGATTTGGATTTGCTCGAG

      430      440      450      460      470      480      490
gBlo t 5  CGATTCAACTACGAAGAGGCTCAAACTCTCAGCAAGATCTTGCTTAAGGATTTCAAGCAACCCGAACAA
gBlo t 21 AAATTCAACTTTGAAGCTGCAATTGGCAACCGGTGATTTGTTGTTGAAGGATCTTAAGGCTCTTCAAAAGC

      500      510
gBlo t 5  AAGTGAACGATATTCAAAACCCAATAA
gBlo t 21 GAGTCCAAGATTCCCAATAG-----

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**FIG E1.** Comparison between the genomic organization of Blo t 5 and Blo t 21. The intron borders are underlined. Identical sequences are shaded with black.