

Genetic polymorphisms and allergenicity of Blo t 5 in a house dust mite allergic Filipino population

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Abstract

Background: Blo t 5, a major allergen from the house dust mite *Blomia tropicalis* had been identified as an important trigger of allergic diseases. Effects of sequence variations to the IgE binding activity of Blo t 5 remain unclear.

Objective: The study determined gene polymorphisms of Blo t 5, expressed the recombinant Blo t 5 allergen in *E. coli* system and determined its IgE reactivity in house dust mite allergic Filipino patients.

Method: Gene polymorphism was determined using Polymerase Chain Reaction and DNA sequencing. Recombinant Blo t 5 was expressed as a GST-fusion protein in *E. coli* and purified by affinity chromatography. IgE-binding activity of 230 age- and sex-matched allergic and non-allergic subjects was subjected to Enzyme-linked Immunosorbent Assay and Western blot analysis.

Results: Five polymorphic variants of Blo t 5 were identified with 26 DNA base and 12 amino acid substitutions thus implying a high degree of sequence diversity. Of the 115 cases, 68.70% and 59.13% showed reactivity to Blo t extract and rBlo t 5 respectively. Total and specific IgE levels of allergic and non-allergic subjects differ significantly ($p < 0.0001$).

Conclusions: Blo t 5 gene exhibits polymorphic variants with predicted amino acid sequences resulting to changes in its IgE epitopes. These polymorphisms may suggest variability of allergenic properties of Blo t 5. The incorporation of the Blo t 5 variants in the panel of diagnostic allergens and immunotherapy is highly recommended.

Keywords: Allergenicity, *Blomia tropicalis*, House Dust Mite, Immunoglobulin, Polymorphism, recombinant Blo t 5

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Introduction

The global prevalence rates of allergic diseases have risen at an alarming rate affecting 20-30% of the population.¹ In the Philippines alone, there is a growing number of people with allergic diseases whose quality of lives has been affected due to their condition.² Allergic reaction is highly characterized by elevated levels of total immunoglobulin E (IgE) and presence of specific IgE to common allergens such as house dust mites (HDMs). HDMs are the most common cause of allergic disorders. Approximately 85% of house dust allergies are mite induced.³ Blo t 5, a major allergen from the house dust mite *Blomia tropicalis* (Blo t) had been identified as an important

trigger of allergic diseases with up to 90% IgE reactivity.³

With the economic costs and social burden associated with allergic diseases, and Blo t 5, as the most important allergen present in the Asian tropics, the study identified gene polymorphisms in Blo t 5, expressed and purified the recombinant form of the said allergen, and evaluated the IgE mediated sensitization of rBlo t 5 among allergic Filipino population. Understanding the role of this allergen in allergic sensitization will lead to a better grasp of the pathophysiology of allergies and in the development of effective and safe therapeutic approaches for HDM allergy.

Methods

Study Design and Subjects

Subjects were selected by purposive, non-randomized method with inclusion and exclusion criteria. Two hundred and thirty serum samples were collected from Filipino unrelated individuals at least 6 to 60 years old, and living in Metro Manila and nearby provinces of Luzon. A total of 115 allergic subjects were chosen based on the modified structured questionnaires set by the International Study of Asthma and Allergy in Childhood (ISAAC) and the International Primary Care Airways Group (IPAG). Cases include individuals with doctor-diagnosed allergic asthma, allergic rhinitis, and atopic dermatitis, either mild or severe, and with a total serum IgE level of >100 IU/ml. Cases were age- and sex-matched with controls. Control subjects include non-allergic individuals, without history of allergy and immediate relatives with allergies. The total serum IgE of the individuals should be <100 IU/ml. The research proposal was subjected to ethical review by the UST Graduate School Ethics Review Committee (Protocol Number GS-2015-166-OC) prior to blood collection. Blood samples were collected, allowed to clot at room temperature and centrifuged for 5 minutes at 5000 rpm to separate the sera which were then stored in aliquots at -20°C until use.

Determination of Genetic Polymorphisms in Blo t 5

Total RNA from cultured *Blomia tropicalis* mites (Siriraj Dust Mite Center, Mahidol University, Bangkok, Thailand) were isolated using NucleoSpin®RNA II Kit (Macherey-Nagel). Approximately 200 live mites were homogenized, lysed, filtered and desalted. The RNA was synthesized into cDNA using the Maxime RT PreMix Kit (iNtRON Biotechnology) with the following RT-PCR conditions: 45°C for 60 minutes for cDNA synthesis and 95°C for 5 minutes for RTase inactivation step. The RT products were used as template for PCR. The full length Blo t 5 gene was isolated by Polymerase Chain Reaction (PCR) with the following set of designed primers, Bt5F: 5' ACAACAATGAAGTTCGCCATC 3' and Bt5R: 5' AAA GTG AAG GAT ATT ACC CAA T 3'. Amplifications were performed in 20 µl PCR reaction mixture with 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72° for 3 minutes and a final elongation step of 72°C for 10 minutes, using GeneAmp PCR System 2400 Thermal Cycler (Perkin Elmer). PCR products were then analyzed on 1% agarose gel using Mupid-exU Submarine Electrophoresis System and were run at 150V for 30 minutes. Gel was visualized using GelDoc (BioRad) and UV Transilluminator. After purification of PCR products using NucleoSpin® Gel and PCR Clean-up Kit (Macherey Nagel), cloning and transformation of the gel purified full-length Blo t 5 cDNA were done by using InstAclone PCR Cloning Kit (Thermo Scientific). The plasmid DNA of the transformed clones were isolated and purified using NucleoSpin® Plasmid DNA Purification Kit (Macherey Nagel). The presence of insert was analyzed by DNA sequencing (Korea Macrogen DNA Sequencing Service). The Blo t 5 nucleotide sequence was compared and aligned with the published Blo t 5 nucleotide sequence using Clustal Omega Multiple Sequence Alignment Software. Phyre2 and USCF

Chimera 9 were used to predict and analyze molecular structures of protein. The physiochemical parameters of the protein sequences were calculated using ProtParam on the ExPASy server.

Recombinant Blo t 5 Allergen Expression and Purification

The Blo t 5 cDNA gene was further ligated into the multiple cloning site of pGEX 4T-1 GST expression vector (GE Healthcare) with BamH1 and Xho1 restriction enzyme sites and transformed in *E. coli* BL21 (GE Healthcare). Correct cloning frames were determined using RE digestion with BamH1 and Xho1 followed by DNA sequencing. The fusion protein was produced by Isopropyl-β-D-thiogalactopyranoside (IPTG) induction and was purified by affinity chromatography using Prepacked GST SpinTrap Columns (GE Healthcare).

Serum IgE Quantitation by Enzyme-Linked Immunosorbent Assay

Enzyme-Linked Immunosorbent Assay (ELISA) was used to determine the total serum and specific IgE concentrations. For total IgE, high-binding microtiter plates (GreinerBio-One) were coated with antihuman IgE capture antibody (Pharmingen) previously diluted with 0.05M sodium bicarbonate followed by overnight incubation at 4°C. The plates were then blocked with 50 µl of 1% Bovine Serum Albumin (BSA) (Sigma-Aldrich) and incubated with diluted serum samples in blocking buffer overnight at 4°C. Serial dilutions of human IgE (Pharmingen) were used as the standard per plate in the calculation of IgE concentration. Biotin conjugated mouse antihuman IgE (Pharmingen) was added followed by the addition of 50 µl of the diluted ExtrAvidin (Sigma-Aldrich). The plates were washed three times with Phosphate Buffered Saline 1X-Tween-20 (PBST). Washing was done after each incubation period using an ELISA washer (BioTek Instruments Inc.). For colorimetric reaction, p-nitrophenyl phosphate (PNPP) substrate (Sigma-Aldrich) was added. The plates were incubated in the dark for at least 30 minutes. Absorbance was read at 405 nm using ELISA reader (BioTek Instruments Inc.).

A standard and experimental set-up were used in ELISA to test for specific IgE reactivity of recombinant Blo t 5. The same protocol for ELISA was followed except that, for the experimental set-up, two antigens were used for coating, rBlo t 5 or Blo t extract instead of the antihuman IgE (Pharmingen). The final concentrations of the recombinant antigen and Blo t extract were 5 µg/ml and 10 µg/ml respectively. Sera from 115 non-allergic subjects were also subjected to ELISA. The IgE reactivity profile of the controls was used as the cut-off value (Mean + 2SD) in determining positive reactions.

Western Blot Analysis

The IgE reactivity of selected serum samples to rBlo t 5 was determined by Western blot analysis. Purified rBlo t 5 was electrophoresed on a 15% Tris-Glycine gel and then electroblotted using Hybond-C Nitrocellulose Membrane (Amersham Life Sciences) using MiniProtean 3 cell (BioRad) at 120V for 60 minutes. The membrane was blocked with 5% skimmed milk diluted with PBS-T and incubated with selected sera diluted with 5X blocking buffer overnight at 4°C.

The membrane was then incubated with biotin-conjugated anti-human IgE (Sigma-Aldrich) for 1 hour followed with peroxidase conjugated ExtrAvidin (Sigma-Aldrich) for another hour. The membrane was washed thrice with PBS-T in between steps. Results were detected by Alkaline Phosphate Color Development Solution (BioRad).

Inhibition Assay

Absorption assay by ELISA was performed to determine the inhibition capacity of Blo t extract against rBlo t 5 and vice versa. A 96-well ELISA plate was coated with the antigens of Blo t extract (10 ug/mL) and rBlo t 5 (5 ug/mL). Six serum samples with high IgE reactivity to Blo t extract and rBlo t 5 as determined by ELISA were selected and used. The allergic sera were preabsorbed with Blo t extract and rBlo t 5 in separate solutions overnight at 4°C followed by human IgE ELISA.

Statistical Analysis

Data were analyzed using statistical softwares SPSSv16, GraphPad Prism 6.0, and MS excel (Microsoft Corp.) Results were considered statistically significant when the p value is <0.05.

Results

Genetic polymorphisms of Blo t 5

The cDNA coding for Blo t 5 consists of 537 bp with ATG start codon at positions 33 to 35,⁵ and a TAA stop codon at positions 435 to 437. Agarose gel electrophoresis of the PCR products of cDNA encoding for Blo t 5 gene and PCR products from purified plasmids migrated at a level which is approximately around 500 bp. A total of six cDNA clones were sequenced and analyzed. The nucleotide sequences aligned using Clustal Omega multiple sequence alignment software showed a number of polymorphic variants in five (Blot5.1, Blot5.3, Blot5.4, Blot5.5, and Blot5.6) out of six clones. Base substitutions were evident in 26 DNA positions (**Figure 1**). At the protein level, polymorphic residues were also found. Amino acid substitutions were seen in 12 positions of the Blo t 5 clones. These polymorphic residues differ from the published sequence of Blo t 5 (**Figure 2**).

Blo t 5 is a helical bundle consisting of three helices in an antiparallel fashion.⁵ It contains four charged residues, Glu76, Asp81, Glu86, and Glu91, all of which have been identified as the four major IgE binding epitope residues.⁶ In Blot5.5, Glu91 was replaced by Glycine. In silico analyses using Phyre 2 protein modeling software and UCSF ChimeraX revealed Blo t 5 clones with triple helical bundles. The physicochemical analyses of the Blo t 5 clones were calculated using ProtParam tool. The surface area and enclosed volume of the surface models ranged from 7289 Å² to 7751 Å² and 7289 Å³ to 7747 Å³ respectively. The molecular weights ranged from 13783.6Da to 13919.7Da which coincide with the approximately 14000Da published molecular weight of Blo t 5. The theoretical pI of the clones ranged from 4.97 to 5.76 with extinction coefficients between 0.214 to 0.216. Aliphatic Index varied between 95.90 and 97.52 while the grand average of hydropathicity (GRAVY) ranged from -0.927 to -0.963 thus are non-polar. Five clones (Blot5.1, Blot5.2, Blot5.3,

Blot5.4, and Blot5.6) showed instability index above 40 thus may suggest potential instability of the protein when expressed. Among the five Blo t 5 clones, Blot5.2 showed 100% homology with the published Blo t 5 sequence thus the said clone was used for recombinant protein expression. However, since Blot5.2 may be unstable, fusing the protein with GST might contribute to increase the stability of the molecule and at the same time increase the yield during the recombinant protein expression. The GST fusion protein system also provided easier purification process of the recombinant protein using Glutathione column.

rBlo t 5 expression and validation

The isolated plasmid, amplified DNA, and the RE digestion products were analyzed separately by agarose gel electrophoresis. The 5401-bp Blo t 5-pGEX-4T-1 recombinant plasmid containing 432-bp insert of the Blo t 5 gene was confirmed in 1% agarose gel. The presence of the Blo t 5 insert in the pGEX-4T-1 expression vector and in-frame cloning were verified by Restriction enzyme (RE) digestion with BamH1 and Xho1, PCR pGEX forward and reverse primers and DNA sequencing. Using the pGEX forward and reverse primers, the purified plasmid was amplified by PCR. Results confirmed the presence of recombinant plasmid pGEX-4T-1 in the E. coli culture and the expected Blo t 5 insert.

Three randomly selected clones containing the Blo t 5-pGEX-4T-1 plasmid were subjected to IPTG Induction followed by SDS-PAGE to determine rBlo t 5 expression. The increased protein levels visible at the expected molecular weight (37kD) on the SDS-PAGE gel after IPTG induction confirmed the successful expression of rBlo t 5. This follows the predicted size of the GST-rBlo t 5 fusion protein which is about 40 kD (26kD GST and 14 kD rBlo t 5). The said recombinant allergen was highly expressed in Blot5.2c (**Figure 3A**). The washed E. coli suspension of IPTG-induced bacterial pellet was lysed using SDS lysis buffer. Presence of this band in the cell lysate, indicated minimal degradation of rBlo t 5. The remaining supernatant was purified by affinity chromatography. The presence of a prominent band at 37kD even after purification clearly showed a purified rBlo t 5 (**Figure 3B**).

Demographic profiles of allergic subjects

Two hundred thirty gender and age matched-paired cases and controls were selected for the study. Majority of the cases suffer from one type of allergy (81.74%) with allergic asthma having the highest percentage (32.17%). Some patients exhibit multiple allergic disorders (18.26%). Most of the cases were 6-20 year old females (38.26%). Allergic patients were mainly from Metro Manila (66.95%) and had family history of allergy (52.84%), were not taking allergy medications (62.61%), were not exposed to second-hand smoke (60.87%), and had household pets (64.35%). The IgE levels of the cases were found between 101.233 to 1259.534 with a mean of 200.124. Results of the Mann Whitney Test revealed that there were no significant differences in the total IgE levels of participants (p>0.05). Furthermore, the Eta correlation coefficients indicate weak associations between the total IgE level and the identified variables.

A	Blot5P	ATGAAGTTCGCCATCGTTCTTATTGCCCTGCTTTGCCCGCTTCGGTTTGGCTCAAGAGCAC	60
	Blot5.1	ATGAAGTTCGCCATCGTTCTTATTGCCCTGCTTTGCCCGCTTCGGTTTGGCTCAAGAGCAC	60
	Blot5.2	ATGAAGTTCGCCATCGTTCTTATTGCCCTGCTTTGCCCGCTTCGGTTTGGCTCAAGAGCAC	60
	Blot5.3	TGGGAGTTCGCCATCGTTCTTATTGCCCTGCTTTGCCCGCTTCGGTTTGGCTCAAGAGCAC	60
	Blot5.4	AATTTGATTCGCCATCGTTCTTATTGCCCTGCTTTGCCCGCTTCGGTTTGGCTCAAGAGCAC	60
	Blot5.5	ATTAAATCGGCCATCGTTCTTATTGCCCTGCTTTGCCCGCTTCGGTTTGGCTCAAGAGCAC	60
	Blot5.6	TTGTTCTTTCGCCATCGTTCTTATTGCCCTGCTTTGCCCGCTTCGGTTTGGCTCAAGAGCAC	60
			
	Blot5P	AAGCCAAAGAACGATGATTTCCGAAACGAATTCGATCACTTGTGATCGAACAGGCCAAAC	120
	Blot5.1	AAGCCAAAGAACGATGATTTCCGAAACGAATTCGATCACTTGTGATCGAACAGGTAAC	120
	Blot5.2	AAGCCAAAGAACGATGATTTCCGAAACGAATTCGATCACTTGTGATCGAACAGGCCAAAC	120
	Blot5.3	AAGCCAAAGAACGATGATTTCCGAAACGAATTCGATCACTTGTGATCGAACAGGCCAAAC	120
	Blot5.4	AAGCCAAAGAACGATGATTTCCGAAACGAATTCGATCACTTGTGATCGAACAGGTAAC	120
	Blot5.5	AAGCCAAAGAACGATGATTTCCGAAACGAATTCGATCACTTGTGATCGAACAGGTAAC	120
	Blot5.6	AAGCCAAAGAACGATGATTTCCGAAACGAATTCGATCACTTGTGATCGAACAGGCCAAAC	120
			
	Blot5P	CATGCTATCGAAAAGGGAGAACATCAATTGCTTTACTTGCACACCAACTCGACGAATTG	180
	Blot5.1	CATGCTATCGAAAAGGGAGAACATCAATTGCTTTACTTGCACACCAACTCGACGAATTG	180
	Blot5.2	CATGCTATCGAAAAGGGAGAACATCAATTGCTTTACTTGCACACCAACTCGACGAATTG	180
	Blot5.3	CATGCTATCGAAAAGGGAGAACATCAATTGCTTTACTTGCACACCAACTCGACGAATTG	180
	Blot5.4	CATGCTATCGAAAAGGGAGAACATCAATTGCTTTACTTGCACACCAACTCGACGAATTG	180
	Blot5.5	CATGCTATCGAAAAGGGAGAACATCAATTGCTTTACTTGCACACCAACTCGACGAATTG	180
	Blot5.6	CATGCTATCGAAAAGGGAGAACATCAATTGCTTTACTTGCACACCAACTCGACGAATTG	180
			
	Blot5P	AATGAAACAAAGACCAAGGAATTGCAAGAGAAAATCATTCCAGAACTTGATGTTGTTTGC	240
	Blot5.1	AATGAAACAAAGACCAAGGAATTGCAAGAGAAAATCATTCCAGAACTTGATGTTGTTTGC	240
	Blot5.2	AATGAAACAAAGACCAAGGAATTGCAAGAGAAAATCATTCCAGAACTTGATGTTGTTTGC	240
	Blot5.3	AATGAAACAAAGACCAAGGAATTGCAAGAGAAAATCATTCCAGAACTTGATGTTGTTTGC	240
	Blot5.4	AATGAAACAAAGACCAAGGAATTGCAAGAGAAAATCATTCCAGAACTTGATGTTGTTTGC	240
	Blot5.5	AATGAAACAAAGACCAAGGAATTGCAAGAGAAAATCATTCCAGAACTTGATGTTGTTTGC	240
	Blot5.6	AATGAAACAAAGACCAAGGAATTGCAAGAGAAAATCATTCCAGAACTTGATGTTGTTTGC	240
			
	Blot5P	GCCATGATCGAAGGAGCCCAAGAGCTTTGGAACGTGAATTGAAGCGAACTGATCTTAAC	300
	Blot5.1	GCCATGATCGAAGGAGCCCAAGAGCTTTGGAACGTGAATTGAAGCGAACTGATCTTAAC	300
	Blot5.2	GCCATGATCGAAGGAGCCCAAGAGCTTTGGAACGTGAATTGAAGCGAACTGATCTTAAC	300
	Blot5.3	GCCATGATCGAAGGAGCCCAAGAGCTTTGGAACGTGAATTGAAGCGAACTGATCTTAAC	300
	Blot5.4	GCCATGATCGAAGGAGCCCAAGAGCTTTGGAACGTGAATTGAAGCGAACTGATCTTAAC	300
	Blot5.5	GCCATGATCGAAGGAGCCCAAGAGCTTTGGAACGTGAATTGAAGCGAACTGATCTTAAC	300
	Blot5.6	GCCATGATCGAAGGAGCCCAAGAGCTTTGGAACGTGAATTGAAGCGAACTGATCTTAAC	300
			
	Blot5P	ATTTTGGAAACGATTCAACTACGAAGAGGCTCAAACTCTCAGCAAGATCTTGCTTAAGGAT	360
	Blot5.1	ATTTTGGAAACGATTCAACTACGAAGAGGCTCAAACTCTCAGCAAGATCTTGCTTAAGGAT	360
	Blot5.2	ATTTTGGAAACGATTCAACTACGAAGAGGCTCAAACTCTCAGCAAGATCTTGCTTAAGGAT	360
	Blot5.3	ATTTTGGAAACGATTCAACTACGAAGAGGCTCAAACTCTCAGCAAGATCTTGCTTAAGGAT	360
	Blot5.4	ATTTTGGAAACGATTCAACTACGAAGAGGCTCAAACTCTCAGCAAGATCTTGCTTAAGGAT	360
	Blot5.5	ATTTTGGAAACGATTCAACTACGAAAGGCTCAAACTCTCAGCAAGATCTTGCTTAAGGAT	360
	Blot5.6	ATTTTGGAAACGATTCAACTACGAAGAGGCTCAAACTCTCAGCAAGATCTTGCTTAAGGAT	360
			
	Blot5P	TTGAAGGAAACCGAACAAAAAGTGAAGGATATTCAAACCCAAATA	405
	Blot5.1	TTGAAGGAAACCGAACAAAAAGTGAAGGATATTCAAACCCAAATA	405
	Blot5.2	TTGAAGGAAACCGAACAAAAAGTGAAGGATATTCAAACCCAAATA	405
	Blot5.3	TTGAAGGAAACCGAACAAAAAGTGAAGGATATTCAAACCCAAATA	405
	Blot5.4	TTGAAGGAAACCGAACAAAAAGTGAAGGATATTCAAACCCAAATA	405
	Blot5.5	TTGAAGGAAACCGAACAAAAAGTGAAGGATATTCAAACCCAAATA	405
	Blot5.6	TTGAAGGAAACCGAACAAAAAGTGAAGGATATTCAAACCCAAATA	405
			

Figure 1. A – Multiple sequence alignment of six cDNA clones (Blot5.1 to Blot5.6) with polymorphic variations (red) compared with the published Blo t 5 gene sequence (Blot5P – green).

	33	34	35	36	37	38	39	41	124	148	355	357	358	417	422	425	426	427	428	430	431	433	434	435	436	437
Blot5P	A	T	G	A	A	G	T	C	T	C	A	G	A	A	T	T	C	A	A	C	C	A	A	T	A	A
Blot5.1	*	*	*	*	*	*	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Blot5.2	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Blot5.3	T	G	*	G	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Blot5.4	*	A	T	T	T	*	A	T	*	T	*	*	*	*	*	*	*	*	*	A	A	C	C	A	*	T
Blot5.5	*	*	T	T	*	A	A	*	C	*	G	A	G	*	*	C	A	*	*	*	*	C	*	A	T	*
Blot5.6	T	*	*	T	T	*	*	T	*	T	*	*	*	G	G	*	*	C	C	*	A	C	C	A	*	*

Blot5	MKFAIVLIACFAASVLAQEHKPKKDDFRNEFDHLLIEQANHAIEKGEHQLLYLQHQDEL	60
Blot5.1	MKFAIVLIACFAASVLAQEHKPKKDDFRNEFDHLLIEQVNHAIKGEHQLLYLQHQDEL	60
Blot5.2	MKFAIVLIACFAASVLAQEHKPKKDDFRNEFDHLLIEQANHAIEKGEHQLLYLQHQDEL	60
Blot5.3	WEFAIVLIACFAASVLAQEHKPKKDDFRNEFDHLLIEQANHAIEKGEHQLLYLQHQDEL	60
Blot5.4	NLIAIVLIACFAASVLAQEHKPKKDDFRNEFDHLLIEQVNHAIKGEHQLLYLQHQDEL	60
Blot5.5	--IAIVLIACFAASVLAQEHKPKKDDFRNESDHLLIEQVNHAIKGEHQLLYLQHQDEL	58
Blot5.6	LLFAIVLIACFAASVLAQEHKPKKDDFRNEFDHLLIEQANHAIEKGEHQLLYLQHQDEL	60
	:*****.*****	
Blot5	NENKSKELQEKIIRELDVVCAMIEGAQGALERELKRTDLNIIERFNYEEAQTLSKILLKD	120
Blot5.1	NENKSKELQEKIIRELDVVCAMIEGAQGALERELKRTDLNIIERFNYEEAQTLSKILLKD	120
Blot5.2	NENKSKELQEKIIRELDVVCAMIEGAQGALERELKRTDLNIIERFNYEEAQTLSKILLKD	120
Blot5.3	NENKSKELQEKIIRELDVVCAMIEGAQGALERELKRTDLNIIERFNYEEAQTLSKILLKD	120
Blot5.4	NENKSKELQEKIIRELDVVCAMIEGAQGALERELKRTDLNIIERFNYEEAQTLSKILLKD	120
Blot5.5	NENKSKELQEKIIRELDVVCAMIEGAQGALERELKRTDLNIIERFNYGRAQTLSKILLKD	118
Blot5.6	NENKSKELQEKIIRELDVVCAMIEGAQGALERELKRTDLNIIERFNYEEAQTLSKILLKD	120
	*****.*****	
Blot5	LKETEQQVKDIQTQ	134
Blot5.1	LKETEQQVKDIQTQ	134
Blot5.2	LKETEQQVKDIQTQ	134
Blot5.3	LKETEQQVKDIQTQ	134
Blot5.4	LKETEQQVKDIQKP	134
Blot5.5	LKETEQQVKDIKTP	132
Blot5.6	LKETEQQVEIPTP	134
	*****.*****	

	1	2	3	31	39	108	109	129	130	132	133	134
Blot5P	M	K	F	F	A	E	E	K	D	Q	T	Q
Blot5.1	*	*	*	*	V	*	*	*	*	*	*	*
Blot5.2	*	*	*	*	*	*	*	*	*	*	*	*
Blot5.3	W	E	*	*	*	*	*	*	*	*	*	*
Blot5.4	N	L	I	*	V	*	*	*	*	*	K	P
Blot5.5	-	-	I	S	V	G	R	*	*	K	*	P
Blot5.6	L	L	*	*	*	*	*	E	E	P	*	P

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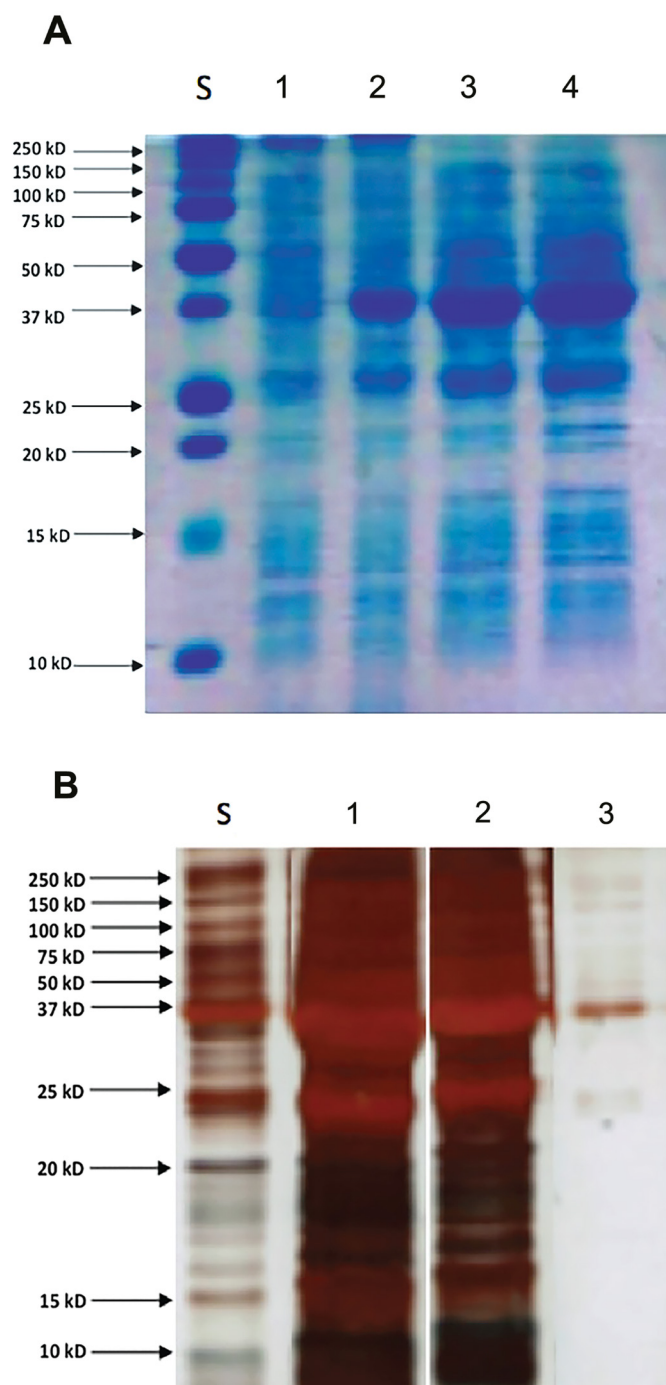


Figure 3. A – Coomassie stained SDS-PAGE gel of expressed rBlo t 5 using the three randomly selected clones. Lane S, protein standard; Lane 1, before induction; Lane 2, after induction of Blot5.2a; Lane 3, after induction of Blot5.2b, and; Lane 4, after induction of Blot5.2c. B – Silver stained SDS-PAGE gel of expressed rBlo t 5. Lane S, protein standard; Lane 1, after induction; Lane 2, cell lysate, and; Lane 3, purified rBlo t 5.

group showed high IgE levels than the non-allergic group. Significant differences ($p < 0.0001$) were also found in the Blo t extract and rBlo t 5 readings of allergic and non-allergic subjects. Expectedly, the atopic cases had generally higher IgE levels.

IgE reactivity of rBlo t 5

Western blot analysis was done in order check for the molecular weight of the IgE-binding proteins present in rBlo t 5 as observed in SDS-PAGE. Eleven serum samples with high

IgE reactivity to rBlo t 5 were selected. All of the patients' IgE reacted to an approximately 37kD protein (**Figure 5A**).

Six serum samples were pre-absorbed overnight with Blo t extract and rBlo t 5 and were introduced onto wells coated with rBlo t 5 and Blo t extract. Results showed that Blo t extract and rBlo t 5 were able to inhibit the binding of rBlo t 5-specific IgEs and Blo t extract present in the sera of allergic cases respectively. Percentage inhibition of Blo t extract against rBlo t 5 ranged from 74.99% to 91.14% with an average of 83.23%.

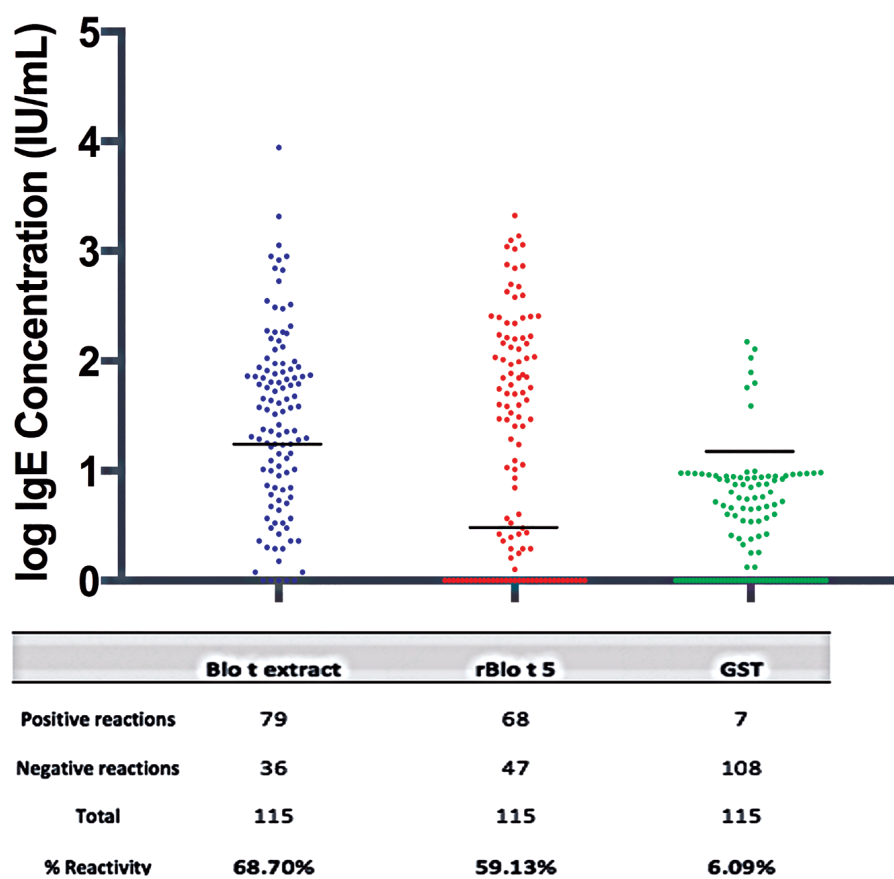


Figure 4. Summary of IgE reactivity of patients to Blo t extract, rBlo t 5, and GST. Horizontal solid lines indicate the cut-off values used in determining positive reactions. Cut off values were computed as mean + 2SD of IgE concentration from non-allergic participants (Blo t extract = 10.715 IU/ml; rBlo t 5 = 3.954 IU/ml; GST = 9.869 IU/ml).

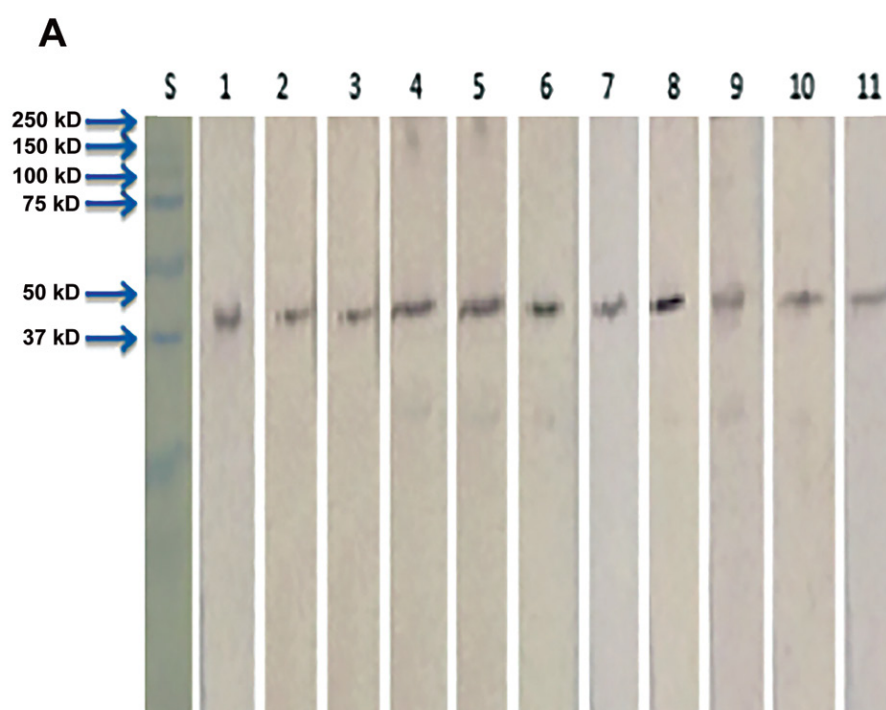


Figure 5. A – Western blot analysis of selected allergic sera sensitized to rBlo t 5. All of the patients' IgE reacted to an approximately 37kD protein. Lane S, protein standard; Lane 1, A006; Lane 2, A009; Lane 3, A025; Lane 4, A026; Lane 5, A028; Lane 6, A061; Lane 7, A064; Lane 8, A071; Lane 9, A092; Lane 10, A100; Lane 11, A104.

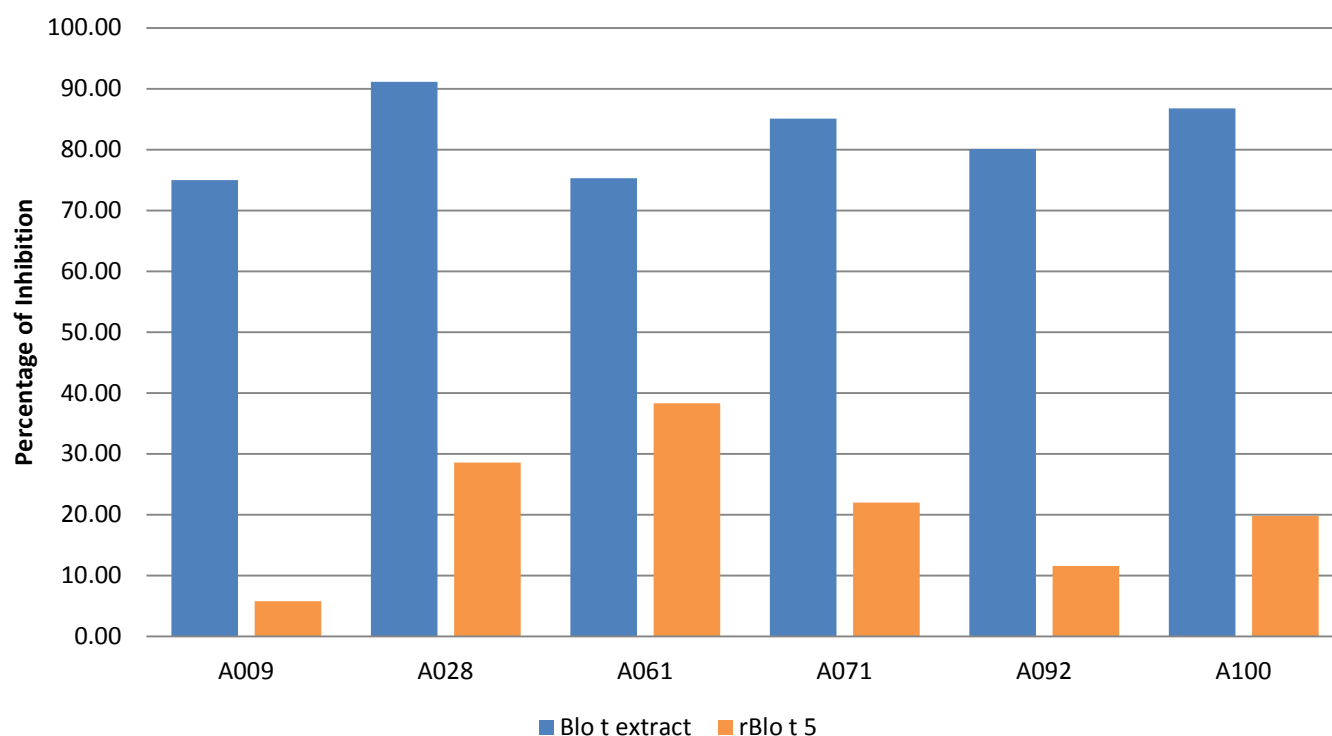


Figure 5. (Continued) B – Graph showing the percentage inhibition assay results of Blo t extract and rBlo t 5.

On the other hand, the percentage inhibition of rBlo t 5 against Blo t extract ranged from 5.77% to 38.30%, the average of which is 21.05% (**Figure 5B**). Results of the inhibition assay suggested that Blo t extract contained additional IgE epitopes that were not recognized by the recombinant allergen.

Discussion

Allergic asthma, rhinitis, and atopic dermatitis are the most common allergic disorders triggered by allergens from HDMs. *Blomia tropicalis* mites constitute a significant portion of the total mite population in tropical and subtropical regions of the world.⁷ Blo t extract was shown to contain at least 30 protein components, and among these, Blo t 5, the 14kD allergen was frequently detected and demonstrated the highest IgE binding frequency.⁸ Identification, classification, and purification of allergens provide understanding of the structural and immunologic aspects particularly how these molecules elicit specific IgE antibody production by the human immune system.⁹ In this study, the Blo t 5 clones exist in several polymorphic variants that may affect allergenicity and T-cell responses. Results of this study are consistent with the NMR study on Blo t 5, consisting of three long alpha helices, forming a coiled-coil, triple-helical bundle.¹⁰ Earlier studies have also shown that Blo t 5 consists of four major IgE binding epitope residues - Glu76, Asp81, Glu86, and Glu91.⁶ All four charged residues are located on the same section that is from 76-91 which is considered to be the major putative linear IgE epitope of Blo t 5.⁶ These IgE epitopes can also be seen on the same location as with the clones except in Blo t 5.5 clone wherein Glu 91 was replaced by Glycine. Replacement of Glu91 by Glycine in Blo t 5.5 clone could have clinical implications. It can

either increase its allergenicity therefore not suitable for use in the diagnosis and treatment of allergic disorders, or it can decrease the allergenicity of the molecule. As such, Blo t 5.5 clone may contribute to the design of a hypoallergenic molecule that can be of potential use for immunotherapy.

Recombinant DNA technology provides the means to produce allergens that are equal to their native counterparts both in structure and immunological properties.¹¹ In this study, the recombinant form of Blo t 5 was successfully expressed in *E. coli* and purified as made evident by the results of SDS-PAGE. The ability to express and purify this recombinant protein in large quantity would allow the potential use of rBlo t 5 as diagnostic and therapeutic reagent for HDM allergy and management.

Allergies are becoming more complex, and patients usually have multiple allergic disorders that is 20-40% of patients with allergic rhinitis are reported to have asthma, 30-90% of patients with asthma have allergic rhinitis, and up to 40% have had or continue to have atopic dermatitis.¹² Results of this study showed the comorbidity of allergic disorders that are consistent with previous studies.^{13,14,15} Susceptibility to allergy is often influenced by several factors such as age, gender, heredity which is by far the most significant, and environmental factors such as exposure second-hand smoke and to household pets.^{16,17,18,19,20}

Allergic disorders are characterized by high levels of IgE which in turn are found to be associated with allergy prevalence and severity.^{21,22,23} About 70-90% of allergic patients worldwide are sensitized to HDMs.²⁴ Several studies were conducted showing Blo t 5 sensitivity. In a study done in Barbados, Blo t 5 sensitivity was present in 46% of 261 subjects.²⁵ Frequencies of sensitization to Blo t 5 in Taiwanese

and Malaysian patients' sera were shown to be 91.8% and 73.5% respectively.²⁶ In a Colombian study, 24% of mite-allergic patients were shown to have IgE-binding to Blo t extract. In the Philippines, Filipinos with respiratory allergies are mostly sensitized to HDMs. About 89% of Filipino allergic patients in Metro Manila were found out to be sensitized to *Blomia tropicalis*.²⁷ Another report on sensitization of selected Filipino allergic subjects showed that 91% were sensitized to Blo t.²⁸ Blo t 5 is recognized by 40-70% of *Blomia tropicalis* sensitized patients whereas rBlo t 5 shows up to 70% IgE reactivity in sensitized asthmatic patients.²⁹ Results of this study are consistent with the earlier studies done that showed significant increased in total and specific IgE levels among allergic individuals than non-atopic controls.

In conclusion, we have identified five Blo t 5 polymorphic variants which may affect allergenicity and T-cell responses. Since Blo t 5 is a major cause of sensitization among allergic Filipino population, the ability to express and purify the recombinant protein in large quantity would allow the potential use of rBlo t 5 as diagnostic and therapeutic reagent for HDM allergy and management. Incorporation of Blo t 5 in the panel of diagnostic allergens and immunotherapy is highly recommended.

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