# ANTIBODIES TO TROPOMYOSIN AND ANTIGENS OF HOUSE DUST MITES IN ASTHMA AND ALLERGIC RHINITIS PATIENTS

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## ABSTRACT

The prevalence of house dust mite (HDM) allergy was determined among the Malaysian population with emphasis to three main species, *Dermatophagoides pteronyssinus*, *D. farinae* and *Blomia tropicalis*. Adult patients with allergy-related diseases, (namely allergic rhinitis and asthma), normal healthy subjects, and asthmatic children were studied for HDM sensitization. In this study, the *in vivo* skin prick test (SPT) was tested on 291 allergic rhinitis and 298 asthmatic patients from the University Malaya Medical Centre, Kuala Lumpur and 30 normal healthy subjects. Of the allergic rhinitis patients, 76% reacted to *D. pteronyssinus* while 72% reacted to *D. farinae*. In the asthmatic group, 80% reacted to *D. pteronyssinus* and 74% reacted to *D. farinae*. In addition, the storage mite, *B. tropicalis* also produced positive SPT results in 66% of the allergic rhinitis patients and 73% in the asthmatics. In allergic rhinitis patients, SPT also revealed sensitivity to other aeroallergens: house dust (40%), cockroach (52%), pollen (10%) and fungal spores (11%). Adult asthmatics also showed higher percentage of reaction to almost all the aeroallergens in SPT, predominantly to HDM species. In addition, seafood also produced quite high percentage of positivity (~30%) in SPT. In 150 asthmatic children, 90% had sensitisation to at least one of the three HDMs and 65% had specific-IgE to all three HDMs. Interestingly, D. *farinae* affected the children more than *D. pteronyssinus;* affecting 85%, followed by *D. pteronyssinus*-specific IgE in 75%, while 74% had IgE against *B. tropicalis*. Monosensitization was detected in 18 (12%) of the children mostly to *D. farinae*. Dust samples from mattresses were found to harbour more mite allergens than the floor and 56% of the number of mattresses studied had Der p 1 compared to 33%, which had Der f 1.

Mite tropomyosin (Der p 10) was selected for its recombinant production and its epitope mapping. The recombinant Der p 10 was obtained by molecular cloning technique through the *E. coli* and the yeast *Pichia pastoris* expression systems. Through sequencing, the deduced amino acid sequence was found to have 98% homology with that of the native Der p 10. *E. coli* expression system produced 1.0 mg/ml purified recombinant GST-Der p 10, whereas the *Pichia* expression system yielded 0.54 mg/ml of protein. *Pichia*-produced Der p 10 showed significant immunogenicity in mice, producing antiserum that could recognize the Der p 10 component in the crude extract. Specific IgG-binding reactivity of the mouse antiserum to these recombinants of Der p 10 could be traced significantly in crude mite extracts and various commercial tropomyosin-containing allergen extracts suggesting that these recombinants of Der p 10 had similar IgG recognition sites as those

found in the native Der p 10 in mite total extracts and other tropomyosin-containing allergens. These Der p 10 recombinants also proved their antigenicity as they were recognizable by shrimp tropomyosin-specific IgG antibody, suggesting the similarity of epitopes on the recombinants to those found on a native tropomyosin protein. *In vivo*, both recombinants also proved functional, eliciting positive reactions comparable with that of crude extracts from tropomyosin-containing allergenic sources such as shrimp and lobster. Subsequently, when tested with ELISA by using sera of Malaysian allergic patients, IgE-binding reactivity with a frequency of about 20-30% was obtained; comparable in frequency and binding strength as observed in 60 Taiwanese allergic patients. This however reflected the failure of these recombinants to emulate the high binding reactivities shown by native tropomyosin (80%) as previously reported.

An investigation of IgE-binding reactivities involving the cloned GST-Der p 10 with another mite tropomyosin, GST-Blo t 10, revealed a high correlation of allergenicity in the allergic subjects. However, low response to Group 10 mite allergens shows that it is not a major allergen in Malaysia. The subsequent evaluation of IgE-binding reactivities involving various recombinant allergenic components in the HDMs, was able to reveal the component-resolved allergenicity, albeit incomplete, in the allergic subjects. Group I and 2 mite allergens, (Der p 1/ Blo t 1 and Der p 2/Blo t 2), identified majority of the sensitized subjects and comparable importance of Group 5 allergens was also discovered.

Both linear and conformational peptides of Der f 10 were synthesized as non-cleavable peptides on pins using the Multipin Peptide Synthesis technique. Subsequently, they proved to be reactive when tested in a modified pinELISA. Pepsacn showed IgE binding at four prominent regions representing the location of B-cell epitopes on Der f 10. Finer mapping using smaller 5-mer peptides confirmed the previously predicted location of epitopes whereby 4 immunodominant epitopes were identified on this allergen; at EVRAL, LQKEV, VDRLE and EDELV showing over 75% IgE-binding reactivity while chimeric peptides identified immunodominant Der f 10 epitopes at KEARMMAEDADRKYDE, ITDEERMDGLENQLKE, EDADRKYDEVARKLAM, DEVARKLAMVEADLER, ERAEERAETGESKIVE and ETGESKIVELEEELRV. Similarly, another allergen, Blo t 5, also constructed as linear synthetic 12-mer peptides overlapping by 5 amino acids, showed distinctive IgE binding reactivities at 4 common regions; at residues 1-12, 56-72, 86-97 to 126-134 of the Blo t 5 protein.

## ABSTRAK

Prevalens alergi hama rumah dikenalpasti di kalangan populasi Malaysia dengan memberi penekanan kepada 3 spesis utama, *Dermatophagoides pteronyssinus*, *D. farinae* dan *Blomia tropicalis*. Penghidap-penghidap rhinitis alergi and asma, subjek-subjek normal, dan kanak-kanak (2-15 tahun) telah dipilih untuk kajian ***sensitization*** terhadap hama rumah. Ujian tusukan kulit (SPT) yang dijalankan melibatkan 291 penghidap rhinitis dan 298 penghidap asthma dari Pusat Perubatan Universiti Malaya, Kuala Lumpur. Hanya 30 subjek normal telah menjalani ujian SPT secara sukarela. Di kalangan penghidap rhinitis alergi, 76% menunjukkan reaksi terhadap *D. pteronyssinus* manakala 72% daripada mereka menunjukkan reaksi terhadap *D farinae*. Dalam kumpulan penghidap asma pula, 80% bereaksi terhadap *D. pteronyssinus* dan 74% terhadap *D farinae*. Selain daripada itu, hama *B. tropicalis* juga menghasilkan keputusan yang positif pada 66% penghidap rhinitis alergi dan 73% di kalangan penghidap asma. Melalui SPT, penghidap rhinitis alergi turut menampilkan kesensitifan terhadap pelbagai alergen lain seperti habuk rumah (40%), lipas (52%), debunga (10%) dan spora kulat (11%). Penghidap asma dewasa turut juga menunjukkan reaksi yang hampir sama. Selain daripada itu, alergen daripada makanan laut seperti ketam, tiram dan udang turut menghasilkan peratus respons SPT-positif yang agak tinggi (~ 30%). Dalam kajian yang melibatkan 150 orang kanak-kanak penghidap asma, 90% daripada mereka adalah sensitif terhadap salah satu daripada hama dalam kajian ini. Daripada bilangan ini, 65% didapati mempunyai IgE-spesifik terhadap ketiga-tiga spesis hama ini. Yang menariknya, *D. farinae* lebih banyak didapati memberi kesan terhadap kanak-kanak (85%) diikuti dengan *D. pteronyssinus* (75%) dan *B. tropicalis* (74%). Kesensitifan tunggal turut dikesan dalam 18 (12%) daripada kanak-kanak ini; kebanyakannya terhadap *D. farinae*. Lebih banyak alergen hama didapati dalam sampel habuk tilam berbanding dengan habuk lantai. Alergen Der p 1 didapati daripada 56% daripada tilam-tilam yang dikaji dan Der f 1 pula dikesan pada 33% tilam-tilam itu.

Di kalangan komponen-komponen protein penyebab alergi pada hama-hama ini, tropomiosin telah dipilih untuk penghasilan rekombinannya dan untuk pemetaan epitopnya. Tropomiosin hama rumah rekombinan (Der p 10) diperolehi melalui teknik pengklonan molekular diikuti dengan sistem pengekspresian bakteria *Escherichia coli* dan yis *Pichia pastoris*. Melalui teknik penjujukan DNA pula, Der p 10 yang dihasilkan oleh *E. coli* didapati mempunyai 98% tahap homologi jika dibandingkan dengan Der p 10 asli. Sistem pengekspresian *E. coli* menghasilkan 1.0 mg/ml rekombinan GST-Der p 10 manakala sistem pengekspresian *Pichia* pula menghasilkan 0.54 mg/ml protein. Der p 10 yang dihasilkan oleh *Pichia* ini menunjukkan ciri keimunogenan yang signifikan pada mencit, dan turut menghasilkan antiserum yang dapat mengecam komponen Der p 10 dalam ekstrak hama yang

asli. Der p 10 rekombinan ini juga dapat dicam oleh antiserum yang dihasilkan terhadap tropomiosin udang. Ini secara tidak langsung menunjukkan bahawa protein rekombinan yang terhasil mempunyai ciri-ciri yang menyerupai tropomiosin asli. Kedua-dua rekombinan Der p 10 ini mampu mengesan 20-30% sampel serum Malaysia yang positif dan keputusan hampir serupa dapat diperhatikan di kalangan 60 penghidap alergi Taiwan. Akan tetapi, walaupun protein-protein rekombinan ini didapati bersifat antigenik, ciri kealergenan tinggi yang dipertunjukkan oleh Der p 10 asli gagal dipamirkan jika dibandingkan dengan kemampuan Der p 10 asli yang dilaporkan dapat mengesan 80% tahap alergi dalam individu-individu beralergi.

Penyiasatan ke atas kereaktifan pengikatan IgE yang melibatkan GST-Der p 10 dan GST-Blo t 10, telah berjaya memperlihatkan korelasi ciri allergenik kedua-dua tropomiosin ini di kalangan subjek-subjek beralergi. Akan tetapi, respons yang rendah terhadapnya menunjukkan bahawa kumpulan alergen ini bukanlah suatu alergen yang bersifat major di kalangan penghidap alergi tempatan. Penilaian seterusnya berdasarkan kereaktifan ikatan IgE yang melibatkan pelbagai komponen alergenik dalam hama yang dihasilkan secara teknik rekombinan, telah berjaya mendedahkan kealergenan hama secara terperinci, walaupun tidak lengkap. Alergen hama Kumpulan 1 (Der p 1/Blo t 1) dan 2 (Der p 2/Blo t 2) telah berjaya mengenalpasti kebanyakan subjek-subjek yang menerima kesan alergen-alergen ini. Alergen hama Kumpulan ke-5 turut didapati memberi kesan yang penting dalam alergi.

Kedua-dua peptida linear dan berkonformasi bagi Der f 10, telah disintesis pada pin-pin melalui teknik 'Multipin Peptide Synthesis' dan diuji dengan teknik ELISA yang diubahsuai untuk mengesan antibodi IgE spesifik pada sampel serum sekumpulan penghidap-penghidap alergi. Kereaktifan pengikatan IgE berfrekuensi tinggi berupaya mendedahkan lokasi-lokasi yang berkemungkinan tinggi menempatkan epitop-epitop sel-B. Pemetaan rapi ke atas epitop-epitop yang disyaki tadi telah mengenalpasti 4 epitop yang dominan secara immunogenik iaitu EVRAL, LQKEV, VDRLE dan EDELV, yang menunjukkan lebih daripada 75% kereaktifan pengikatan IgE. Peptida-peptida kimerik yang didapati reaktif pula ialah KEARMMAEDADRKYDE, ITDEERMDGLENQLKE, EDADRKYDEVARKLAM, DEVARKLAMVEADLER, ERAEERAETGESKIVE dan ETGESKIVELEEELRV. Allergen Blo t 5 dalam bentuk peptida sintetik linear 12-mer dengan pertindihan 5 amino asid telah dapat mengesan 4 jujukan yang berkemungkinan menempatkan epitop-epitop iaitu di amino asid pertama hingga ke-12, ke-56 hingga ke-72, ke-86 hingga ke-97 dan ke-126 hingga ke-134 pada jujukan protein Blo t.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ABTS | 2,2'-azino-bis[3-ethylbenz-thiazoline-6-sulfonate] |
| Amp | Ampicillin |
| bp | base-pair |
| BSA | bovine serum albumin |
| cDNA | complementary DNA |
| C-terminal | carboxyl terminal |
| DIC | Diisopropylcarbodiimide |
| DMF | N,N-dimethylformamide |
| dNTP | any mixture of the four deoxynucleotide-5' -triphosphates: dATP, dCTP, dGTP, dTTP. |
| DNA | Deoxyribonucleic acid |
| dOD | δ-optical density |
| EDTA | ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| Fmoc | 9-fluorenylmethyloxycarbonyl |
| GST | Glutathione-*S*-transferase |
| HOBt | N-hydoxybenzotriazole |
| HRP | Horseradish peroxidase |
| IgA | Immunoglobulin A |
| IgE | Immunoglobulin E |
| IgG | Immunoglobulin G |
| IPTG | Isopropyl-β-thiogalactopyranoside |
| LB | Luria-Bertani broth |
| LLB | Low salt Luria broth |
| kb | kilobase |
| kDa | kiloDalton |
| MW | Molecular weight |
| mM | milliMolar |
| N-terminal | amino terminal |
| OPD | Ortho-phenylenediamine |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| *Pfu* | ***Pyrococcus furiosus*** |
| PMSF | Phenylmethylsulfonyl fluoride |
| pNPP | ρ– Nitrophenyl phosphate |
| psi | per square inch |
| rpm | revolutions per minute |
| SDS | Sodium dodecyl sulphate |
| SPT | Skin prick test |
| TAE | Tris-acetate-EDTA |
| *Taq* | ***Thermus aquaticus*** |
| TBS | Tris-buffered saline |
| TEMED | N,N,N',N'-tetramethyl-ethylenediamine |
| Tw20 | Tween-20 [polyoxyethylensorbitanmonolaurat] |
| v/v | volume per volume |
| w/v | weight per volume |

## AMINO ACID ABBREVIATIONS

| Amino acid | Three letter code | Single letter code |
|---|---|---|
| Alanine | Ala | A |
| Cysteine | Cys | C |
| Aspartic acid | Asp | D |
| Glutamic acid | Glu | E |
| Phenylalanine | Phe | F |
| Glycine | Gly | G |
| Histidine | His | H |
| Isoleucine | Ile | I |
| Lysine | Lys | K |
| Leucine | Leu | L |
| Methionine | Met | M |
| Asparagine | Asn | N |
| Proline | Pro | P |
| Glutamine | Gln | Q |
| Arginine | Arg | R |
| Serine | Ser | S |
| Threonine | Thr | T |
| Valine | Val | V |
| Tryptophan | Trp | W |
| Tyrosine | Tyr | Y |

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# INTRODUCTION

## Introduction

Many aspects in the field of allergy have been studied since nearly a century ago. Initially the various manifestations of allergy were observed; followed by the prevalence studies of allergic diseases. The search for the causal factors of allergies and even patterns of allergen distribution (e.g. pollen and spores) were studied to understand allergy. Gradually, more refined and detailed studies were carried out on the immunology of allergy, to further understand the pathways and mechanisms of allergic reactions.

In the 5th Malaysian Congress of Allergy and Immunology held this year in Kuala Lumpur, Assoc. Prof. Dr. Ranbir Kaulsay of University Putera Malaysia, predicted that by the time Malaysia achieved developed status in the year 2020, almost half of the population would develop some form of allergy ( *The Star*, May 2004).

The hypothesis in this study is that mite tropomyosin is a major allergen among the allergens of house dust mites, on the basis of its high allergenicity, affecting ~80% of atopic patients as reported by Aki *et al.*, (19954a). The house dust mites (HDM) from the *Dermatophagoides* species have been the main contributor of allergens in Malaysia. In the early 1970's, a preliminary study of the patients visiting hospitals with allergy complaints found the HDM to be one of the main causal factors of allergy symptoms such as allergic rinitis and asthma. Today, nearly thirty years later, this study seeks to find out if HDM still maintain their prevalence in contributing allergens. Another recently discovered species of mite in Malaysian fauna, the *Blomia tropicalis* was also investigated. Previously well known as a storage mite, we seek to address whether *B. tropicalis* has become a 'domestic' dust mite, as reported widely in many region of the world. It is of great interest that if specific IgE antibodies to *B. tropicalis* can be detected in the allergic patients, therefore, the role of this species in allergy will thus be verified. Most importantly, this will actually alter the idea that *B. tropicalis* is only an occupational threat, i.e. to workers pertaining to storage or agriculture.

Currently, although antihistamines and steroids are of great help to alleviate the allergy symptoms, there is still a need for more effective treatments to induce long-term tolerance to allergens or immunity from allergies. One such step in this direction is allergen vaccination or immunotherapy, which modifies the patient's allergic response by gradually exposing him/her to high levels of a particular allergen and thus helps the body to build a tolerance to it so that, eventually, the immune reaction towards that allergen is reduced. A novel form of treatment is to use purified allergens in liquid form, introduced into the body through a series of subcutaneous injections or sublingual drops. This underlines the role of protein chemistry in not only isolating allergens for diagnostic purposes but also for the above-mentioned treatment. With the advent of proteomics, the separation and characterisation of the multiple allergens in allergy-causing species have been diligently reported since the 80's, yielding a cascade of information on the allergenic components in terms of their contents, functions, and allergenicity. Group 1 and 2 mite allergens Der p 1 and Der p 2 emerged as the most prevalent allergens affecting most atopic individuals, thus granting them the term "major allergens". Many researchers then began to report about other components of allergens, in the quest for the complete repertoire of antigens or allergens in *Dermatophagoides pteronyssinus.* Among these antigens, Der f 10, the mite tropomysin, was singled out to be further studied. This emphasis was due to the fact that it was initially reported as a novel protein (previously mag44), able to elicit ~80% allergenicity in the Japanese population. Its common occurrence in many crustaceans or seafood, and it being a lesser-studied allergen compared to other major mite allergens in Group 1 and 2; therefore justifies its appropriate attention in this study.

Recombinant DNA (rDNA) techniques greatly contribute to in-depth studies of allergen characterisation. Through the cloning of its cDNA, and expression of the putative allergens via heterologous host systems (e.g. bacteria or yeast), purified recombinant

allergens can be used in *in vivo* and *in vitro* studies in place of the scarce, and often-impure natural allergens. In this study, the production of recombinant mite tropomyosin (rDer p 10), via the bacterial and yeast expression systems was therefore investigated.

When the mapping of both B-cell and T-cell epitope(s) on antigens became a prime activity in immunology, many allergens too, were scrutinised for the exact fragment(s) or peptide(s) (in the case of protein allergens) which can be recognised by specific antibodies leading up to the whole allergic reactions. Elucidating the properties of epitope(s) on allergens is important for understanding the structure-function relationship of antigen-antibody interaction. Most importantly, the correct identification of antigenic epitopes will greatly aid the diagnosis and prognosis of a disease, allergy included. Ultimately, the identification of epitope(s) on allergens as recognised by antibodies particularly IgE, is useful in pinpointing events underlying the genetics and development of allergy. Epitope determination also contributes to the advancement of immunotherapy in the ultimate quest of eradicating or desensitising allergy.

In this study, the literature review of allergy is duly introduced in **Chapter 1**, which includes the definition of allergy, allergens and the epidemiology of allergy based on previous studies in Malaysia. This chapter also presents the literature review of relevant topics pertaining to house dust mites, recombinant allergens and peptide synthesis. The various materials and methods used in this study are described in **Chapter 2**. Subsequently, in **Chapter 3**, the current house dust mite allergenicity was first determined among Malaysian allergic patients, through Skin Prick Tests followed by data profiles of *in vitro* investigation through ELISA method. On the sideline, investigations of dust sampling, salivary IgA in comparison to the presence of IgE in the serum, and severity of asthma in relation to HDM sensitivity were also carried out and results were then discussed here. The recombinant production of mite tropomyosin through both prokaryotic and eukaryotic expression systems were compared in **Chapter 4** and subsequently, together with some important recombinant proteins representing the almost complete repertoire of HDM

allergens, the IgE reactivity profiles to these Der p recombinants were determined in a selected mite-allergic population by specific IgE detection through ELISA. Thus a component-resolved study of HDM allergenicity in both allergic rhinitis and asthma patients was investigated and compared in **Chapter 5**. Lastly, **Chapter 6** describes the use of synthetic peptides of two allergens, mite tropomyosin (Der f 10) and Blo t 5 in pepscans involving pinELISA for epitope mapping. Finally, conclusions and future recommendations were aptly made at the end of this study. Abbreviations of frequently used terms were listed, together with the amino acids. References and appendices have been included to complement the presentation of this study.

**Objectives of present study**

The specific objectives of this study are:-

1. to investigate the prevalence of HDM allergenicity among Malaysians. These included atopic patients with asthma and allergic rhinitis; healthy subjects and asthmatic children while the main species of HDM studied were *Dermatophagoides pteronyssinus* and *D. farinae.* This study aims at obtaining a sensitization profile of atopic patients in Malaysia, including knowledge of reactivity to a storage mite, *Blomia tropicalis.*

2. to evaluate the antibody profiles against the HDM allergens. The specific antibodies studied were IgE, IgG and salivary IgA; and the allergen components were in the form of recombinant proteins to develop diagnostic concepts that may help determine an improved selection of allergens for future skin prick tests or immunotherapy in our local clinics or hospitals.

3. to clone the cDNA encoding Der p 10, a major allergen from *Dermatophagoides* spp., and express Der p 10 as a recombinant protein through the prokaryotic host system of *Escherichia coli* and the eukaryotic host system of *Pichia pastoris.*

4. to investigate the B-cell response to mite tropomyosin synthetic peptides in the determination of antigenic determinants through epitope mapping i.e. by immuno-scanning the mite tropomyosin molecule, for antibody binding sites by the pinELISA method using the synthesized peptides.