CHARACTERIZATION OF MAJOR AND UNIQUE *BLOMIA TROPICALIS* MITE ALLERGENS

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List of Publications

Paper

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2. **Yeoh SM**, Kuo IC, Wang DY, Liam CK, Sam CK, De Bruyne JA, Lee BW, Cheong N, Chua KY. "*Dermatophagoides pteronyssinus* and *Blomia tropicalis* sensitization profiles among Malaysian and Singaporean subjects" at the 5th Asia Pacific Congress of Allergology and Clinical Immunology, The 7th West Pacific Allergy Symposium, 12-15 October 2002, Seoul, Korea.
3. **Yeoh SM**, Cheong N, Chua KY. "Monoclonal antibody specific for a unique allergen from *Blomia tropicalis*." at the 7th NUS-NUH annual scientific meeting 2-3 October 2003, Singapore.

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Summary

Sensitization profiles of rhinitis, non-rhinitis healthy subjects and asthmatic subjects (from Singapore and Malaysia respectively) against three major mite allergens Der p 1, Der p 2 and Blo t 5 were studied using enzyme-linked immunosorbent assay (ELISA). The sensitization profile of rhinitis subjects to the domestic mite allergens used in this study was as follow: *Blo t* extract +: 91 / 124 (73%); Blo t 5 +: 62 / 124 (50%); *Der p* extract +: 61 / 124 (49%); Der p 1 +: 53 / 124 (43%); Der p 2 +: 45 / 124 (36%). The non-rhinitis healthy subjects' sensitization profile was as follows: *Blo t* extract +: 60 / 105 (57%); Blo t 5 +: 24 / 105 (23%); *Der p* extract +: 38 / 105 (36%); Der p 1 +: 14 / 105 (13%); Der p 2 +: 17 / 105 (16%). Study on Malaysian subjects showed that 39% of the adult patients with asthma were sensitized to Der p 1; 32% to Der p 2; 37% to Blo t 5. The corresponding sensitization profiles in the asthmatic children were 57% to Der p 1, 39% to Der p 2 and 90% to Blo t 5. Therefore, these allergens are important sensitizing agents and should be included in component-resolved diagnosis of mite sensitization.

Besides that, a unique allergen from *Blomia tropicalis* (*Blo t*), Blo t 19 was identified through cDNA library screening. Blo t 19 is a small (around 7 kD) and cysteine-rich protein. Recombinant form of Blo t 19 was a minor allergen. Sequence analysis revealed that Blo t 19 had high sequence (76%) similarity with *Ascaris suum* antibacterial factor (ASABF). Blo t 19 is a possible CSαβ-type peptide based on sequence comparison with ASABF. Blo t 19 is also the first protein not identified among nematodes to be having a very high amino acid sequence similarity with ASABF.

A Blo t 19-specifc monoclonal antibody which was useful for detection of Blo t 19 in western blot and ELISA was successfully raised using a combination of DNA immunization and protein boost in mice. However, the purification procedures of native Blo t 19 using this monoclonal antibody remain elusive.

It was observed that conventional method of immunizing the mice failed to induce antibody against Blo t 19. Besides that, the strain of mice could influence the chance of inducing antibody against Blo t 19.

In short, this study revealed the sensitization profiles of rhinitis and asthmatics subjects in this region; identified a unique *Blo t* allergen, Blo t 19, and successfully raised a monoclonal antibody that was useful in detecting Blo t 19.

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Chapter 1

1 Introduction

1.1 *Background of the study*

The prevalence of allergic diseases such as asthma and allergic rhinitis increased significantly in the early 90s (Sears, 1997; ISAAC, 1998 (a-b)); Linneberg *et al*., 1999; Linneberg *et al*., 2000; Strannegård & Strannegård, 2001; Babu & Arshad, 2003). Although several studies from Italy, Switzerland and Australia recently indicated that at least the increasing trend has stopped in the respective studied populations, these allergic diseases remain an important health issue in the population (Ronchetti *et al*., 2001; Braun-Fahrlander *et al*., 2003; Toelle *et al*., 2004). Allergic diseases such as asthma and allergic rhinitis not only cause a drop in the quality of life of the people affected by them but could also be fatal at times in the case of asthma (Baraniuk, 1997; Holgate, 1999). Various epidemiological studies around the world showed that the prevalence of allergic diseases ranged from around 2% to 30% in some countries (ISAAC, 1998 (a); Janson *et al*., 2001). The difference in the prevalence could be due to genetic predisposition and environmental factors such as life style (Barnes & Marsh, 1998; von Mutius *et al*., 1998; Howard *et al*., 1999; Zhang *et al*., 1999; Cookson & Moffatt, 2000; Janson *et al*., 2001; Strannegård & Strannegård, 2001; Cookson, 2002; Yazdanbakhsh *et al*., 2002).

Among the environmental factors, the presence or absence of allergens in the surroundings is a determining factor whether one will be sensitized and/or develop an allergic disease (Platts-Mills & Chapman, 1987; Lau *et al*., 1989; Sporik *et al*., 1990). Domestic mites (include house dust mites (HDM) (family Pyroglyphidae) and storage

mites (family Acaridae, Glycyphagidae and Chortoglyphidae)), especially *Dermatophagoides pteronyssinus* (*Der p*), *Dermatophagoides farinae* (*Der f*), and *Blomia tropicalis* (*Blo t*) are major sources of allergens that cause allergic asthma and rhinitis (Voorhorst *et al*., 1967; Platts-Mills & Chapman, 1987; Platts-Mills & de Weck, 1989). Due to the fact that domestic mites are very common in indoor environment around the world (Ho, 1986; Hurtado & Parini, 1987; Arlian *et al*., 1992; Zhang *et al*., 1997; Colloff, 1998 (a)), people are easily exposed to mite allergens and sensitized to them (Lau *et al*., 1989; de Groot *et al*., 1990; Sporik *et al*., 1990). Therefore, it is important to study the mite sensitization in order to better understand and control the rise of allergic diseases.

The advent of advances in molecular biology allowed various allergens to be identified and cloned from domestic mites (Thomas & Smith, 1998; Thomas & Smith 1999; Thomas *et al*., 2002; Kawamoto *et al*., 2002 (a)). Currently, around 19 different groups of allergens had been identified from domestic mites (Thomas *et al*., 2002; Kawamoto *et al*., 2002 (a); Mills *et al*., 1999; Lim *et al*., 2002; Yi *et al*., 2002; Lee *et al*., 2002; Kawamoto *et al*., 2002 (b), Flores *et al*., 2003; Mora *et al*., 2003; Cheong *et al*., 2003 (a-b); Saarne T *et al*., 2003; http://www.allergen.org/List.htm; Weber *et al*., 2003). The identification of individual allergens are important for better diagnosis and treatment of mite allergy.

Although there were a number of studies on the prevalence of mite sensitization (Woodcock & Cunnington, 1980; Ho *et al*., 1995; Leung *et al*., 1997; Baratawidjaja *et al*., 1999; Chew *et al*., 1999 (a)), mite crude extracts were used as the reagents. The usefulness of recombinant and purified domestic mites allergens as reagents for sensitization studies had not been fully evaluated.

Monoclonal antibody is a valuable reagent in mite allergy study (Chapman *et al*., 1987; Luczynska *et al*., 1989; Härfast *et al*., 1992; Yunginger & Adolphson, 1992; Ovsyannikova *et al*., 1994; Ferrándiz *et al*., 1995; Shen *et al*., 1995; Shen *et al*., 1996; Ferrándiz *et al*., 1997; Peng *et al*., 1998; Tsai *et al*., 2000; Labrada *et al*., 2002; Park *et al*., 2002; Parvaneh *et al*., 2002; Trombone *et al*., 2002; Ramos *et al*., 2003). Various methods had been employed to generate monoclonal antibody (Köhler & Milstein, 1975; Smith, 1985; McCafferty *et al*., 1990; Clackson *et al*., 1991; Marks *et al*., 1991). These include the conventional fusion of spleenocytes from immunized mice and myeloma cells to generate monoclonal antibody producing hybridomas and unconventional phage display method (Köhler & Milstein, 1975; Smith, 1985; McCafferty *et al*., 1990; Clackson *et al*., 1991; Marks *et al*., 1991).

1.2 *Overall objectives of the study*

The overall objectives of the study were as follows:

- To study the sensitization profiles of rhinitis and non-rhinitis healthy subjects in Singapore.
- To study the sensitization profiles of adult asthmatics and children asthmatics in Malaysia.
- To isolate a unique allergen from Blo t using cDNA library screening.
- To raise monoclonal antibodies specific against the unique allergen, Blo t 19, identified in cDNA library screening.

1.3 *Overall significance of the study*

Firstly, this study is one of the first studies reporting the sensitization profiles of rhinitis and asthmatics patients in South East Asia using individual mite allergens

Der p 1, Der p 2 and Blo t 5. This study showed that Blo t 5 sensitization was generally more prevalent among these subjects compared to Der p 1 and Der p 2. This further established the importance of *Blo t* allergens in relation to allergic diseases in this part of the world. More importantly, it also showed that these three allergens are important reagents in component-resolved diagnosis of mite sensitization.

Secondly, the identification of Blo t 19 from *Blo t* mite contributed to the effort of identification of a more complete and representative spectrum of allergens from domestic mites. Blo t 19 is also the first protein not identified among nematodes to be having a very high amino acid sequence similarity with an antibacterial factor from *Ascaris suum*, *Ascaris suum* antibacterial factor (ASABF). ASABF could be considered distantly related to insect defensins (Dimarcq *et al*., 1998). ASABF has antibacterial activity against a range of bacteria and yeast (Zhang *et al*., 2000).

Thirdly, this study also successfully raised a Blo t 19-specific monoclonal antibody. Nonetheless, it also showed that generation of monoclonal antibody against Blo t 19 was difficult despite different methods employed: DNA immunization, protein immunization through different routes. Further optimization of the immunization schedule and methods of immunization could probably increase the chance of obtaining the monoclonal antibody of choice.

Chapter 2

2 Literature review

2.1 *Allergy & allergic airway diseases*

Allergy is a complex phenomenon of the human immune response. The term "allergy" was first introduced by von Pirquet in 1906 to describe biological responses which could lead to either immunity or allergic disease (Kay, 2001). Today, the term "allergy" is used almost interchangeably with IgE-mediated allergic responses (Kay, 2001). Nevertheless, effort has been made to standardize the definition of this term (Johansson *et al*., 2001). The more acceptable definition for allergy is: allergy is a series of hypersensitive reactions caused by the Th2-skewed immune system of the body (Figure 1) (Holgate, 1999; Holt *et al*., 1999; Johansson *et al*., 2001). In certain cases, these reactions could be also cell-mediated, such as in the case of contact dermatitis where sensitized lymphocytes played a major role (Johansson *et al*., 2001).

In IgE-mediated allergy, elevated levels of IgE in the patient's sera induce allergic responses. These include wheezing, rhinoconjunctivitis, gastrointestinal symptoms, lesions in the skin (eczema) and anaphylaxis. IgE-mediated allergic diseases include allergic asthma, allergic rhinitis, allergic conjunctivitis, atopic eczema / dermatitis syndrome (AEDS) and urticaria (Johansson *et al*., 2001).

Substances (mainly proteins, and some carbohydrate) that induce immunological response once encountered by the body are known as antigens. Allergens are antigens that induce allergic responses (Johansson *et al.*, 2001). The important allergens found so far came mainly from domestic mites, grass pollen, birch pollen and animal dander

(Voorhorst *et al*., 1967; Kay, 2001; Thomas *et al*., 2002). Allergens generally come into contact with humans through the mucosal surfaces (Holgate, 1999).

The prevalence of allergy and allergic diseases around the world is generally on the rise in the recent years due to change in environment factors and life style (Robertson *et al*., 1991; Åberg *et al*., 1995; Sears, 1997; Boner *et al*., 1998; Lundbäck, 1998; ISAAC, 1998 (a-b); von Mutius *et al*., 1998; Linnerberg *et al*, 1999; Linneberg *et al*., 2000; Kay, 2001). Change of life style towards more "westernized" one and improved in cleanliness have been linked with the increase in the prevalence of allergy and allergic diseases (von Mutius *et al*., 1998; Strannegård & Strannegård, 2001; Yazdanbakhsh *et al*., 2002)

The diagnosis of allergy is performed by measuring the free or cell-bound IgE using *in vivo* and *in vitro* diagnosis methods. *In vivo* diagnoses of allergy is skin prick tests (SPT) whereas *in vitro* methods, include radioallergosorbent assay (RAST), enzyme-linked immunosorbent assay (ELISA) and Pharmacia CAP system (Wide *et al*., 1967; Johansson *et al*., 1999; Bousquet *et al*., 2001).

2.1.1 Immunoglobulin E (IgE)

Immunoglublin E (IgE) (originally known as γE-Globulin (Ishizaka *et al*., 1966 (a)) or IgND (Johansson, 1967) when first discovered) was discovered between 1966 and 1967 (Ishizaka *et al*., 1966 (a), Ishizaka *et al*., 1966 (b), Johansson, 1967). It was first reported to be associated with asthma by Johansson (Johansson, 1967) and at the same time an assay (radioallergosorbent test (RAST)) was developed to detect this immunoglobulin in the sera (Wide *et al*., 1967). Later in 1968, this new class of immunoglobulin was officially named IgE (Bennich *et al*., 1968).

Normal, non-atopic individuals have very low IgE titre. Normal individuals usually have less than 100 KU / l (1 U = 2.4 ng) of serum IgE. If an adult has over

100-150 KU / l of IgE, then he/she is considered above normal (Bousquet *et al*., 2001). The typically median level of total IgE is 200-400 kU / l in normal atopic diseases and a level of more than 1000 kU / l suggests various complications (Aalberse, 2000). The half-life of IgE in sera is less than two days while IgE bound to mast cells in the skin can last for around 10 days (Platts-Mills T, 2001).

The synthesis of IgE is shown in Figure 1. Allergen-specific IgE is synthesize as a result of the interactions of B cell – Th2 cell – mast cells / basophils, upon the presentation of allergen to Th2 cell by antigen presenting cell (APC) (Yssel *et al*., 1998; Corry & Kheradmand, 1999; Holgate, 1999; Holt *et al*., 1999).

Various studies have shown the association of IgE level (total IgE or allergen-specific IgE) to allergic diseases (Burrows *et al*., 1989; Sears *et al*., 1991; Kotaniemi-Syrjänen *et al*., 2003). For instance, Sears and colleagues showed that in asthmatic children, IgE levels are associated with physician-diagnosed asthma and bronchial hyperresponsiveness (BHR) (Sears *et al*., 1991). Nonetheless, the correlation of total IgE levels to disease is less compared to allergen-specific IgE (Aalberse, 2000).

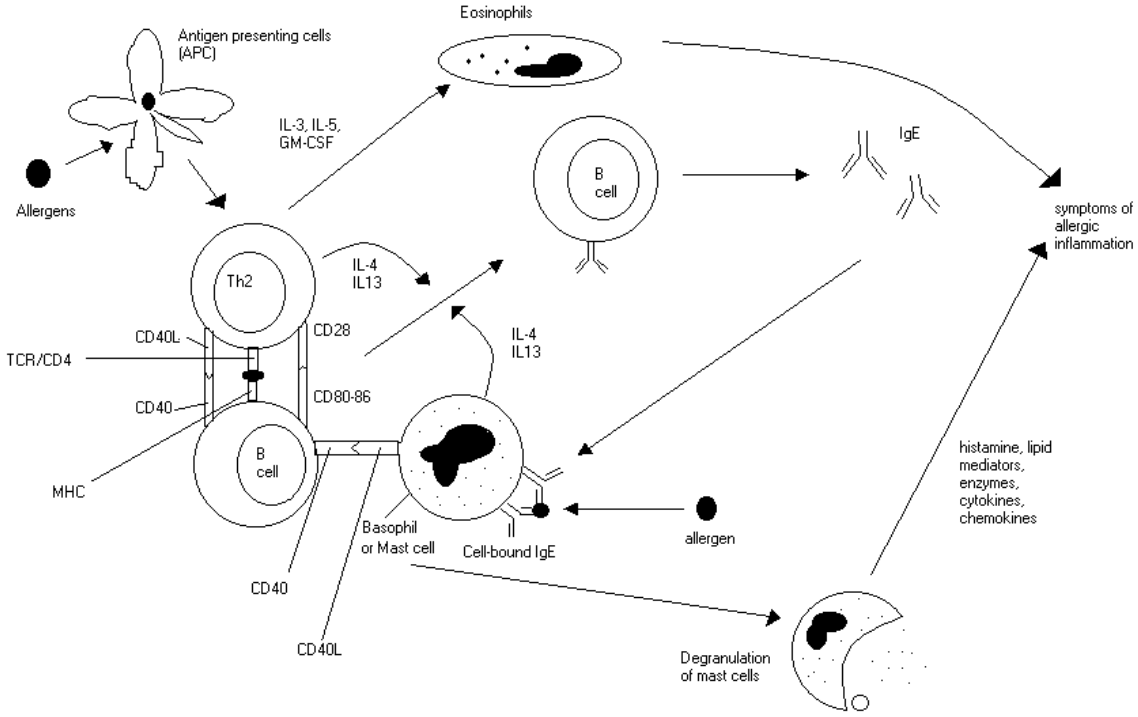

Figure 1: Regulations of IgE synthesis and allergic responses (adapted from Yssel *et al*., 1998; Corry & Kheradmand, 1999; Holgate, 1999; Holt *et al*., 1999).

2.1.2 Allergic rhinitis

Allergic rhinitis is a term used to describe hypersensitivity of the nose caused by immunological reactions of the body which usually resulting in the production of antigen-specific IgE (Johansson *et al.*, 2001). Allergic responses involved in allergic rhinitis include itch, sneeze, congestion, drip, fatigue and dysfunction (Baraniuk, 1997). The allergens that cause allergic rhinitis are inhaled allergens which include pollen, acarids, animal dandruff and fungi (Baraniuk, 1997; Passàli *et al*., 2001).

House dust mites and storage mites played a major role in allergic rhinitis. Domestic mites that are most commonly found in homes in various parts of the world are *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae* and *Blomia tropicalis* (Ho, 1986; Hurtado *et al*., 1987; Platts-Mills & de Weck, 1989; Arlian *et al*.,

1992; Malainual *et al*., 1995; Puerta *et al*., 1996 (a); Arlian *et al*., 1999; Chew *et al*., 1999 (b); Arlian, 2000; Sopelete *et al*., 2000; Passàli *et al*., 2001). Majority of allergic rhinitis patients are sensitized to mites (Bousquet *et al*., 2001).

Various studies have shown that allergic rhinitis and asthma often coexist (Lombardi *et al*., 2001; Linneberg *et al*., 2002). Some studies managed to show that allergic rhinitis is a risk factor for asthma (Leynaert *et al*., 1999; Guerra *et al*., 2002; Linneberg *et al*., 2002, Torén *et al*., 2002).

2.1.3 Allergic asthma

A worldwide study on the prevalence of asthma has yield wide range of differences (ISAAC, 1998 (a)). The ISAAC study, performed in 56 countries, showed that the prevalence of asthmatic symptoms among children aged 13-14 years ranged from less than 5% in Indonesia to over 30% in the United Kingdom (ISAAC, 1998 (a)). An additional report by the same study, focusing specifically on the prevalence of asthma worldwide (ISAAC, 1998 (b)) had showed similar findings: the prevalence of asthma was up to 15-folds differences between countries (ISAAC, 1998 (b)). In this report by ISAAC, study subjects aged 6-7 years were also included besides the 13-14 years group. The prevalence rate ranged from 1.6-3.0% in Albania, Estonia, Ethiopia, Indonesia, Iran, Poland, Russia, South Korea and Uzbekistan to 20.7-28.2% in Australia, New Zealand, Oman, Peru, Singapore and the United Kingdom (ISAAC, 1998 (b)). Although the prevalence of asthma varies from country to country, its burden is important and should not be overlooked.

Besides having a high prevalence, asthma is a common illness that could seriously affect the quality of life of the sufferers. Both wheezing at night and night cough could disturb sleep.

An individual with allergic asthma has the tendency to develop airway hyperresponsiveness (AHR) in the lung, airway inflammation and allergic sensitization (elevation in the antigen-specific IgE titres) (Corry, 2002). When IgE is involved in the pathogenesis of asthma, the disease is known as allergic asthma. Allergic asthma can be differentiated from non-allergic asthma based on skin prick tests results (Romanet-Manent *et al.*, 2002). It is also known that allergic asthma is strongly linked to genetic factors (Zhang *et al*., 1999; Cookson & Moffatt, 2000).

A study performed by Romanet-Manent *et al*. clearly showed the clinical differences between allergic asthma and non-allergic asthma (Romanet-Manent *et al*., 2002). According to the study, allergic patients were significantly younger than non-allergic patients and there was a female-biased in non-allergic asthma (more female compared to male). Besides that, allergic asthma was more influenced by the change of seasons compared to non-allergic asthma. Although in the study, no significant difference was observed on the prevalence of rhinitis among the allergic and non-allergic asthmatics, the authors observed that there was a trend of higher rate of sneezing in allergic asthmatics (Romanet-Manent *et al*., 2002). The other important observation by the same study was that non-allergic asthmatics tend to have more serious asthma symptoms compared to allergic asthmatics.

On the physiological level, Walker *et al*. showed that allergic asthmatics had elevated levels of interleukin-4 (IL-4) and interleukin-5 (IL-5) whereas the non-allergics had higher levels of interleukin-2 (IL-2) and IL-5. The elevation in IL-4 in allergic asthmatics resulted in the elevation of IgE in the sera of these patients (Walker *et al*., 1992).

Besides inducing the synthesis of IgE (Figure 1), IL-4 is also involved in another immunological pathway which resulted in airway hyper-responsiveness and

goblet-cell metaplasia by acting directly on the airway smooth muscle and epithelium (Corry, 2002). Nevertheless, the role of IL-4 in this pathway is not as important compared to another interleukin – interleukin-13 (IL-13) (Wills-Karp *et al*., 1998; Grünig *et al*., 1998). In fact, it has been shown recently that IL-13 causes airway hyperreactivity and mucus over-production in asthma (Kuperman *et al*., 2002)

There has been an increasing number of reports, showing that allergic asthma and allergic rhinitis are a uniform airway disease (Bousquet *et al*., 2001; Guerra *et al*., 2002; Lundblad, 2002; Linneberg *et al.*, 2002).

2.2 *Sensitization: a general definition*

Antigen specific IgE secreted by plasma cells binds with the Fcε receptor (FcεRI) on the surface of mast cells and blood basophils (Baraniuk, 1997). These "sensitized" cells upon second encounter with the same allergen undergo degranulation, releasing active mediators such as histamine that exert biological effects on surrounding tissues (Kuby, 1992). Sensitization can also be defined as a primary response to allergens which primarily induce the differentiation of $CD4^+$ T cells to T helper 2 (T_H2) cells (Figure 1) (Valenta 2002; Constant *et al*., 2000). The first definition takes into account the downstream process of sensitization only whereas the latter definition gives a more thorough picture from the upstream to downstream of the whole process of sensitization. The main indication that one is sensitized is the presence of allergen-specific IgE in the sera and / or on mast cells and basophils. Therefore, a subject is classified as sensitized if he / she is positive in skin prick tests and / or allergen-specific IgE is detected in his / her serum (Platts-Mills & de Weck, 1989).

2.2.1 Prevalence of mite sensitization

The prevalence of mite sensitization in the general population varies from country to country and is under the influence of different climatological conditions (Murray *et al*., 1985; Burney *et al*., 1997). A study conducted on randomly selected individuals across 37 centres in 16 countries by The European Community Respiratory Health Survey revealed that the prevalence of *Der p* sensitization ranged from 6.7% to 35.1% (Burney *et al*., 1997). Nevertheless, the prevalence of mite sensitization was still among the highest compared to other allergens in the same study (Cat: 2.7%-14.8%; Grass: 8.1%-34.6%; *Cladosporium* spp: 0.3%-13.6%) (Burney *et al*., 1997). Mite sensitization is more prevalent among subjects from humid areas compared to subjects from "dry areas" (Murray *et al*., 1985). This was largely due to the fact that domestic mites generally survive better in humid conditions (Arlian *et al*., 1998 (a-b); Bousquet *et al*., 2001). Therefore, exposure to mites is more possible and frequent in humid areas.

The prevalence of mite sensitization among patients with allergic diseases offered a different picture. Various studies on the mite sensitization among these patients showed that the prevalence was very high (70-90%) and correlated well with the disease state (Rizzo *et al*., 1993; Stanaland *et al*., 1994; Ho *et al*., 1995; Droste *et al*., 1996; Ferrándiz *et al*., 1996; Nelson *et al*., 1996; Boulet *et al*., 1997; Leung *et al*., 1997; Tsai *et al*., 1998 (a); Baratawidjaja *et al*., 1999; Mori *et al*., 2001; Verini *et al*., 2001). Mite sensitization has been recognized as a risk factor for the development of allergic diseases, such as allergic asthma and allergic rhinitis (Sporik *et al*., 1990; Peat *et al*., 1996; Leung *et al*., 1997; Boner *et al*., 1998; Lynch *et al*., 1998; Scalabrin *et al*., 1999; Chou *et al*., 2002; del Giudice *et al*., 2002; Wong *et al*., 2002). Association studies conducted across 22 countries on around 140000 individuals also showed good

association between sensitization to mite and bronchial responsiveness (Janson *et al*., 2001). The association of allergenic sensitization with the development of allergic diseases, especially asthma and rhinitis is well accepted now (Burrows *et al*., 1989; Yssel *et al*., 1998; Oettgen & Geha, 2001). This is to be noted that not all sensitization leads to disease (Wahn, 2000). Other factors such as genetic factor, environment and lifestyle, are also involved (Sporik *et al*., 1999; von Mutius *et al*., 1998; Kurz *et al*., 2000; Wahn, 2000; Kay, 2001; Strannegård & Strannegård, 2001; Kauffmann *et al*., 2002; Yazdanbakhsh *et al*., 2002).

2.2.2 Crude extracts versus recombinant / purified allergens

There are basically two choices of allergens in sensitization study, the first one being the crude extracts and the second being recombinant or purified allergens. Studies of mite sensitization in the population have traditionally been carried out using crude extracts prepared from the allergen sources (either prepared in house or obtained commercially) (Pepys *et al*., 1967; Woodcock & Cunnington., 1980; Murray *et al*., 1985; Puerta *et al*., 1991; Rizzo *et al*., 1993; Stanaland *et al*., 1994; Ho *et al*., 1995; Droste *et al*., 1996; Ferrándiz *et al*., 1996; Nelson *et al*., 1996; Boulet *et al*., 1997; Burney *et al*., 1997; Leung *et al*., 1997; Tsai *et al*., 1998 (a); Baratawidjaja *et al*., 1999; Chew *et al*., 1999 (a); Kuo *et al*., 1999; Yi *et al*., 1999; Mori *et al*., 2001; Verini *et al*., 2001; Court *et al*., 2002; Jaén *et al*., 2002; Müsken *et al*., 2002). *Dermatophagoides pteronyssinus* (*Der p*) mite crude extracts has traditionally been used as a representation of mite allergens in mite sensitization studies (Pepys *et al*., 1967; Woodcock & Cunnington., 1980; Murray *et al*., 1985; Puerta *et al*., 1991; Droste *et al*., 1996; Ferrándiz *et al*., 1996; Nelson *et al*., 1996; Boulet *et al*., 1997; Burney *et al*., 1997; Leung *et al*., 1997; Baratawidjaja *et al*., 1999; Chew *et al*., 1999 (a); Kuo *et al*., 1999; Verini *et al*., 2001; Court *et al*., 2002; Jaén *et al*., 2002; Müsken *et al*., 2002).

This was largely due to the reason that *Der p* mite dominates the indoor mite populations in various parts of the world and was long known to be a major source of allergens (Voorhorst *et al*., 1967; Maunsell *et al*., 1967; Arlian *et al*., 1992; Malainual *et al*., 1995; Colloff, 1998 (a); Arlian *et al*., 1999; Chew *et al*., 1999 (b)). Nonetheless, lately, the importance of other domestic mites was recognized and crude mite extracts from other mites such as *Dermatophagoides farinae* (*Der f*) and *Blomia tropicalis* (*Blo t*) have been included along with *Der p* (Rizzo *et al*., 1993; Stanaland *et al*., 1994; Ho *et al*., 1995; Ferrándiz *et al*., 1996; Nelson *et al*., 1996; Tsai *et al*., 1998 (a); Baratawidjaja *et al*., 1999; Mori *et al*., 2001).

The use of recombinant or purified allergens as reagents to study sensitization profile is relatively new compared to the use of crude extracts. Recombinant allergens were only available with the advancement in recombinant DNA technology. Nonetheless, various investigators have started to realize the advantages of such allergens confer in their studies (Valenta & Kraft, 1995; Kraft *et al*., 1999; Heiss *et al*., 1999; Johansson *et al*., 1999; Tsai *et al*., 1998 (a); Valenta & Vrtala, 1999; Valenta *et al*., 1999 (a); Kronqvist *et al*., 2000; Kazemi-Shirazi *et al*., 2002; Valenta, 2002 (a-b); Simpson *et al*., 2003). Recombinant allergens not only gave more consistent results in sensitization studies but also are valuable reagents for immunotherapy and vaccination (Kraft *et al*., 1999; Valenta *et al*., 1999 (b); Kazemi-Shirazi *et al*., 2002).

As a matter of fact, a combination of recombinant or purified allergens could potentially replace the use of crude extract in sensitization study, as demonstrated by Laffer *et al*. using recombinant pollen and birch allergens (recombinant pollen allergens: Phl p 1, Phl p 2, Phl p 5; recombinant birch allergen: Bet v 2) where they showed that the combination of these allergens accounted for 94.5% (173 / 183) of grass pollen specific IgE (Laffer *et al*., 1996). Similar result was also obtained by a

study conducted by Valenta *et al*. where 97 / 98 grass pollen-allergic patients were identified using 2 recombinant pollen allergens (Phl p I; Phl p V) and 1 grass recombinant allergen (profilin) (Valenta *et al*., 1992). However, another study by Niederberger *et al*. only managed to detect 59% of grass pollen-allergic subjects (Niederberger *et al*., 1998). This could be due to cohort differences and experimental design. Besides that, the choice on the panel of recombinant allergens could also influence the sensitivity of the detection. If some major allergens are being left out of the panel, obviously the sensitivity of the test will be reduced as well. Logically, a combination of purified native allergens also showed good results: over 90% of extract positive subjects were detected (van Ree *et al*., 1998; van Ree *et al*., 1999). To date, no similar studies were conducted on recombinant or purified mite allergens.

2.3 *Domestic mites*

Domestic mites consist of various free-living mites that are found living in houses. This includes house dust mites (HDM) (family Pyroglyphidae) and storage mites (family Acaridae, Glycyphagidae and Chortoglyphidae) (Colloff *et al.*, 1992; Platts-Mills *et al*., 1992). Pyroglyphidae mites are also known as nidicolous mites as most of them lived in the nests of birds and mammals (Warner *et al*., 1999). Acaridae and Glycyphagidae mites are known as storage mites mainly because they are often found in large numbers in barns, silos, and other habitats where agricultural products are stored (Warner *et al*., 1999). They are well characterized morphologically (Voorhorst *et al*., 1967; Colloff *et al*., 1992; Colloff, 1998 (b)). Human and animal skin scales are the major food for Pyroglyphidae mites while decaying plants and similar products are food for Acaridae and Glycyphagidae mites (Warner *et al*., 1999). Nonetheless, a study by Naspitz *et al*. showed that both HDM (*Dermatophagoides pteronyssinus* and *Euroglyphus maynei*) and storage mite (*Blomia tropicalis*) could be

detected in dust samples collected from children scalps, indicating not only Pyroglyphidae mites but also Glycyphagidae mites could feed on human dandruff (Naspitz *et al*., 1997). Domestic mites that were reported to be prevalent in various home environments around the world were mainly from family Pyroglyphidae and Glycyphagidae (Hurtado *et al*., 1987; Platts-Mills & Chapman, 1987; Arlian *et al*., 1992; Puerta *et al*., 1996 (a); Mariana *et al*., 1996; Arlian *et al*., 1999; Chew *et al*., 1999 (b); Arlian, 2000; Sopelete *et al*., 2000).

In general, domestic mites grow best under hot (above 23°C) and humid (80% relative humidity) conditions (Platts-Mills & Chapman, 1987; Arlian *et al*., 1998 (a-b); Bousquet *et al*., 2001). Example of exception to the rule is *Euroglyphus maynei* which had been shown to be unable to survive at relative humidity higher than 65% (Arlian *et al*., 1998 (a)). Domestic mites require high humidity to survive (Hart, 1998) because their main source of water supply comes from water vapour. Only at humidity of 65-70%, sufficient water could be extracted from the air in their surroundings (Arlian, 1992).

Although the term "domestic mites" was proposed to be used to describe house dust mites and storage mites collectively (Platts-Mills *et al*., 1992) and has since been used by various investigators (Naspitz *et al*., 1997; Müsken *et al*., 2002), throughout the literature, the term "dust mite" was commonly used to describe storage mites as well (Eriksson *et al*., 1998; Eriksson *et al*., 1999; Gafvelin *et al*., 2001). This text will adopt the term "domestic mites" to describe house dust mites and other non-pyroglyphidae mites collectively and reserve the term HDM for Pyroglyphidae mites only for future discussion.

There exist two schools of thought on the taxonomy of domestic mites in the literature (Arlian & Platts-Mills., 2001; Colloff, 1998 (b); Colloff & Spieksma, 1992;

Olsson & van Hage-Hamsten, 2000). The former system was mainly followed by Colloff *et al*., and Olsson *et al*. (Colloff 1998 (b); Colloff & Spieksma, 1992; Olsson & van Hage-Hamsten, 2000) while the latter was used by Arlian and colleagues (Arlian & Platts-Mills, 2001). Nonetheless, the differences between the two systems were not significant. The differences between both systems were outlined in Table 1 (Table 1), using *Blomia tropicalis* as an example. Since more information was available on the system used by Colloff (Colloff, 1998 (b); Olsson & van Hage-Hamsten, 2000), further discussion on the taxonomy of domestic mites will be based on this system.

	Arlian *et al*., 2001	**Colloff, 1998 (b)**
Kingdom	Animalia	Animalia
Phylum	Arthropoda	Arthropoda
Subphylum	Chelicerata	Chelicerata
Class	Arachnida	Arachnida
Subclass	-	Acari
Order	Acari	Acariformes
Infraorder	-	Sarcoptiformes
Suborder	Astigmata	Astigmata
Superfamily	-	Glycyphagoidea
Family	Echymyopodidae	Glycyphagidae
Genus	*Blomia*	*Blomia*
Species	*tropicalis*	*tropicalis*

Table 1: Differences between two classification systems, using Blomia tropicalis as an example (based on Arlian *et al*., 2001; Colloff, 1998 (b); Olsson & van Hage-Hamsten, 2000).

As shown in Table 1, domestic mites belong to Class Arachnida, indicating that the mites are more closely related to spiders than to insects. Mites under the suborder Astigmata lack specialized respiratory organs (Colloff & Spieksma, 1992).

Though house dust had been shown to cause skin reactions in asthmatic patients as early as in 1921 and 1922 respectively (Kern, 1921; Cooke, 1922), it was not until around 1967 when *Der p* was identified to be the major allergen contributor in house dust (Voorhorst *et al*., 1967; Maunsell *et al*., 1967). From then onwards, various

studies had been performed to study mites in the house dust and their relevance to allergy. It is now known that domestic mites that play major roles in allergy are mainly from family Pyroglyphidae: *Dermatophagoides pteronyssinus* (*Der p*), *Dermatophagoides farinae* (*Der f*), and from family Glycyphagidae: *Blomia tropicalis* (*Blo t*). To date, around 19 different groups of allergens had been identified in domestic mites (Thomas *et al*., 2002; Kawamoto *et al*., 2002 (a); Table 2).

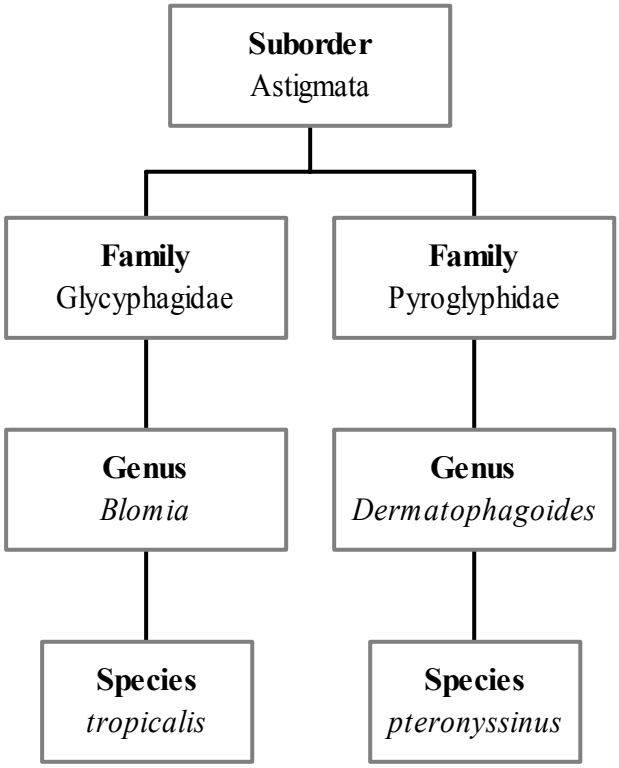

Figure 2: Taxonomy of *Blomia tropicalis* and *Dermatophagoides pteronyssinus*

2.3.1 *Dermatophagoides pteronyssinus* (*Der p*)

Mites of the genus *Dermatophagoides* was first described by Bogdanov in 1864 (Colloff, 1998 (b)). Morphologically, as with other mites under the family Pyroglyphidae (Figure 2), *Der p* has "fingerprint" pattern of striations on its body. According to Colloff (Colloff 1998 (b)), mites in the genus *Dermatophagoides* are characterized by the difference in length of setae present on their body, and the absence of tegmen. *Dermatophagoides* mites mainly survive in nature on skin debris

and dandruff of animals and human beings. They live permanently in house dust and thus the term house dust mite (HDM) has often been used to describe collectively all the mites that are found consistently in house dust. Nevertheless, only six out of the thirteen species of mites from family Pyroglyphidae are distributed worldwide (Platts-Mills & de Weck, 1989; Colloff, 1998 (b)). These include *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Hirstia domicola*, *Malayoglyphus intermedius, Sturnophagoides brasiliensis.* and *Euroglyphus maynei* (Ho, 1986; Platts-Mills & de Weck, 1989; Colloff 1998 (b)).

Der p was the earliest known mite to have a role in causing allergy (Voorhorst *et al*., 1967; Maunsell *et al*., 1967). It was later known that mite faeces are a major source of house dust allergens and the allergen in the faecal pellet was mainly Der p 1, one of the major allergen from *Der p* (Tovey *et al.*, 1981).

2.3.2 *Blomia tropicalis* (*Blo t*)

Storage mite, *Blo t*, belongs to the Family Glycyphagidae (Figure 2). Morphologically, like other mites from family Glycyphagidae, Blo t has a smooth cuticle, body covered with minute papillae, and long serrated dorsal setae (Colloff & Spieksma, 1992; Colloff, 1998 (b)). It is prevalent in the tropical region (Chew *et al*., 1999 (b); Puerta *et al*., 1996 (b)). *Blo t*, like other mites species, survives best under high humidity and temperature (Bousquet *et al*., 2001).

The significance of this mite in allergy has been extensively studied in the recent years by various investigators (Arlian *et al*., 1993; Stanaland *et al*., 1994; Arruda *et al*., 1995; Puerta *et al*., 1996 (b); Caraballo *et al*., 1997; Chew *et al*., 1999 (a); Shek *et al*., 1999; Yi *et al*., 1999; Yi *et al*., 2002; Angus *et al*., 2002; Ramos *et al*., 2001; Medeiros *et al*., 2002; Ramos *et al*., 2003). As a matter of fact, various investigators have shown that *Blo t* is an important source of allergens in the tropical

and subtropical regions (Caraballo *et al*., 1993; Arruda *et al*., 1995; Puerta *et al*., 1996; Leung & Lai, 1997; Platts-Mills *et al*., 1997; Zhang *et al*., 1997; Fernández-Caldas, 1997; Arruda *et al.*, 1997; Caraballo *et al*., 1997; Kuo *et al*., 1999; Yi *et al*., 1999; Chew *et al*., 1999 (b); Yi *et al.*, 1999; Arlian & Platts-Mills, 2001; Ramos *et al*., 2001; Medeiros *et al*., 2002).

2.4 *Allergens from domestic mites*

2.4.1 Overview of mite allergens

The importance of dust sensitization in bronchial asthma was first noticed by Dr. Richard Kern in 1921 (Kern, 1921). It was not until around 1964-1967 when Voorhorst and co-workers showed that dust sensitization was actually mainly sensitization to allergens from HDM, *Der p* (Voorhorst *et al.*, 1967). Maunsell *et al*. and Pepys *et al*. also obtained similar findings (Maunsell *et al*., 1967; Pepys *et al*., 1967). In addition, Maunsell *et al*. and Pepys *et al*. also showed the presence of sensitization to storage mites and other mites (*Glycyphagus domesticus*, *Acarus siro* in both studies and *Tyrophagus putrescentiae* in Maunsell *et al*.'s study) among subjects tested (Maunsell *et al*., 1967). Nonetheless, the sensitization frequencies to these mites in their findings were relatively small compared to sensitization to *Dermatophagoides* mites. These were some of the earliest available data on sensitization to storage mites. Besides, one interesting point to note was in Pepys *et al*.'s study, they used *Dermatophagoides culinæ* (now known as *Dermatophagoides farinae*) instead of *Dermatophagoides pteronyssinus* because they obtained similar results in skin prick tests using both extracts on 14 patients (Pepys *et al*., 1967).

Over the years, various allergens from domestic mites were identified and characterized (Table 2). In the process, these allergens were divided into groups based

on their sequence similarity, biochemical composition and molecular weight (King *et al.*, 1994; Liebers *et al*., 1996; Arlian *et al*., 2001). As a general rule in naming allergens, the name of the allergen must use the following format: the first three letters of the genus are followed by the first letter of the species and the allergen number (King *et al*., 1994). For instance, group 1 allergens are called Der p 1 (group 1 allergen from ***Der****matophagoides* ***p****teronyssinus*), Der f 1 (group 1 allergen from ***Der****matophagoides* ***f****arinae*) and Blo t 1 (group 1 allergen from ***Blo****mia* ***t****ropicalis*) (Chapman & Platts-Mills, 1980; Heymann *et al.*, 1986; Mora *et al*., 2003). The various groups of allergens have been, over the years, extensively reviewed by Thomas *et al*. and Kawamoto *et al*. (Thomas & Smith, 1998; Thomas & Smith 1999; Thomas *et al*., 2002; Kawamoto *et al*., 2002 (a)).

Group	Mites[+]	Molecular weight	Biochemical function	IgE binding (%)
1	*Der p, Der f, Der m, Der s, Eur m, Blo t, Pso o*	25000	Cysteine protease	60-100
2	*Der p, Der f, Der s, Eur m, Lep d, Tyr p, Gly d, Pso o*	14000	Unknown (HE1 homologue)	80-100[#]
3	*Der p, Der f, Der s, Eur m, Blo t*	25000-30000	Trypsin	50-100
4	*Der p, Blo t, Eur m*	57000	α-Amylase	30-46
5	*Der p, Blo t, Lep d*	15000	Unknown	50-70
6	*Der p, Der f, Blo t*	25000	Chymotrypsin	40
7	*Der p, Der f, Lep d*	25000	Unknown	50
8	*Der p*	26000	Glutathione-S-transferase (GST)	40
9	*Der p*	30000	Collagenolytic serine protease	90
10	*Der p, Der f, Blo t, Lep d*	32930 (Lep d) 37000	Tropomyosin	13-95
11	*Der f, Blo t*	96000	Paramyosin	80
12	*Blo t*	14000	Unknown, Chitin-binding protein?	50
13	*Blo t, Lep d, Aca s*	15000	Fatty acid-binding protein	11-23
14	*Der f, Der p, Eur m*	177000 (variable)	Vitellogenin/apolipophorin-like, Mag3	39-70
15	*Der f*	62500	98000 Chitinase	70
16	*Der f*	55131	Gelsolin	18-47
17	*Der f*	~53000	Ca-binding EF protein	35
18	*Der f*	60000	Chitinase	54
19	*Blo t*	7000	Anti-microbial peptide	10
*	*Der f, Lep d*	50007 (*Lep d*)	Heat-shock protein 70 (*Der f*) α-tubulin (*Lep d*)	12 (α-tubulin)

[#] = IgE binding for Pso o 2 undetermined; * = undesignated

Table 2: List of groups of allergens identified thus far in domestic mites

[+]**Note:** ***Aca s: Acarus siro; Blo t: Blomia tropicalis; Der p: Dermatophagoides pteronyssinus; Der f: Dermatophagoides farinae; Der m: Dermatophagoides microceras; Der s: Dermatophagoides siboney; Eur m: Euroglyphus maynei; Gly d: Glycyphagus domesticus Lep d: Lepidoglyphus destructor; Pso o: Psoroptes ovis; Tyr p: Tyrophagus putrescentiae***

2.4.1.1 **Group 1 allergen**

Group 1 allergen (Table 2) was first identified by Chapman *et al*. in *Der p* (Chapman & Platts-Mills, 1980), later in *Dermatophagoides microceras* (*Der m*) (Lind, 1986); in *Dermatophagoides farinae* (*Der f*) by Heymann *et al*. and Dilworth *et al*. (Heymann *et al*., 1986; Dilworth *et al*., 1991); in *Euroglyphus maynei* (*Eur m*) by Kent *et al*. (Kent *et al*., 1992); in *Dermatophagoides siboney* (*Der s*) (Ferrándiz *et al*., 1995) and recently in Blo t (Mora *et al*., 2003; Cheong *et al*., 2003 (a)). Besides that, group 1 allergen has also been identified in sheep scab mite, *Psoroptes ovis* (Lee *et al*., 2002). Allergens from group 1 family have 221 to 223 amino acid residues and have a calculated molecular weight of 25000 daltons. (Chua *et al*., 1988; Dilworth *et al*., 1991; Chua *et al*., 1993; Mora *et al*., 2003).

The characteristics of Der p 1 and Der f 1 have since been extensively studied (Lind, 1985; Lind, 1986; Chapman *et al*., 1987; van der Zee *et al*., 1988; Chua *et al*., 1993; Hewitt *et al.*, 1995; Hewitt *et al*., 1997; Gough *et al*., 1999). Sequence analysis revealed that group 1 allergens are cysteine proteases (Chua *et al*., 1988; Ando *et al*., 1991; Heymann *et al*., 1986). Later study on Der p 1 revealed that it has a mixture of cysteine and serine protease activity (Hewitt *et al*., 1997). The enzymatic function of group 1 allergens could have a role to play in their allergenicity as studies have shown that group 1 allergens could cleave the human IgE receptor CD23 (Hewitt *et al.*, 1995). The cleavage of CD23 might directly enhance the synthesis of IgE (Hewitt *et al*., 1995). In addition, a study by Gough *et al*. also showed that mice immunized with proteolytically active Der p 1 had significant elevation in their total IgE compared to mice immunized with Der p 1 inhibited by cysteine protease inhibitor E-64 (Gough *et al*., 1999). It was hypothesized that the cysteine protease activity of Der p 1 could have

destabilized the microenvironment of target tissues to one that was pro-allergic and thus induced the allergic responses (Gough *et al*., 1999). Although it was generally accepted that group 1 allergens are cysteine proteases, Blo t 1, which was recently cloned, as a cysteine protease, has yet to be proven to possess cysteine protease activity although having significant sequence identity with cysteine proteases from mites and other organisms (Mora *et al*., 2003).

With the exception of Pso o I which had only been shown to bind IgE in infested sheep (Lee *et al*., 2002), other group 1 allergens have been shown to be allergenic in humans (Chapman & Platts-Mills, 1980; Heymann *et al*., 1986; Ferrándiz *et al*., 1995; Mora *et al*., 2002). In fact, group 1 allergen, especially Der p 1 and Der f 1 are major allergens for humans (Chapman & Platts-Mills, 1980; Heymann *et al*., 1986). For Der p 1, Chapman and colleagues showed that three quarters of IgE antibody against *Der p* mite extract was directed against Der p 1 (Chapman & Platts-Mills, 1980). Similar finding was also observed by Heymann and co-workers for Der f 1 where 29 / 42 (69%) children and 55 / 63 (87%) adults who were allergic to *Der f* had IgE against Der f 1 (Heymann *et al*., 1986). Recently, Mora and co-workers also showed that recombinant Blo t 1 bound IgE from 13 / 21 (62%) Blo t mite extract positive patients (Mora *et al*., 2003). Although Der p 1 could account for most of the mite allergic subjects, there were clearly some who were not accounted for. This has driven other investigators to search for other allergens from the mites, which brings about the different groups of allergens identified so far.

2.4.1.2 **Group 2 allergens**

Group 2 allergen (Table 2) from Der p was first isolated by Lind using chromatography and was originally named as Dp X (Lind, 1985). The cDNA of Der p 2 was subsequently identified by Chua *et al*. using IgE plaque immunoassay (Chua *et*

al., 1990). It was later named as Der p 2 under the guidelines by the WHO IUIS Allergen Nomenclature Subcommittee (King *et al*., 1994). Following the discovery of Der p 2, other group 2 allergens in other mites were also identified subsequently by various investigators: Der f 2 (Yasueda *et al*., 1986; Holck *et al*., 1986) in *Dermatophagoides farinae*; Lep d 2 (initially known as Lep d 1 when first described) (Ventas *et al*., 1992; van Hage-Hamsten et al., 1992; Varela *et al*., 1994; Schmidt *et al*., 1995) in *Lepidoglyphus destructor*; Der s 2 in *Dermatophagoides siboney* (Ferrándiz *et al*., 1995); Tyr p 2 (Eriksson *et al*., 1998) in *Tyrophagus putrescentiae*; Eur m 2 (shown to exist in Eur m extract by Morgan *et al*., 1997 but cloned by Smith *et al*., 1999) in *Euroglyphus maynei*; Gly d 2 (Gafvelin *et al*., 2001) in *Glycyphagus domesticus* and Pso o 2 (Temeyer *et al*., 2002) in *Psoroptes ovis*. Group 2 allergen is a 14 000 protein and shows various degrees of similarity with each other. For instance, Der p 2, Der f 2 and Eur m 2 were shown to share more than 80% amino acid sequence identity (Chua *et al*., 1996; Smith *et al*., 1999) whereas Lep d 2, Tyr p 2 shared lesser identity with Der p 2 (around 40% identity) (Gafvelin *et al*., 2001). Lep d 2 and Gly d 2 shared up to 79% sequence identity indicating they are more phylogenetically linked to each other than to other mites (Gafvelin *et al*., 2001). The function of group 2 allergens is largely unknown but there were indications that group 2 allergens might have similar function as epididymis-specific human HE1 gene product based on sequence analysis (Thomas & Chua, 1995).

Group 2 allergens identified thus far, except for Pso o 2, are all considered major allergens (Lind, 1985; Yasueda *et al*., 1986; Heymann *et al*., 1989; Morgan *et al*., 1997; Lynch *et al*., 1997; Eriksson *et al*., 1998; Tsai *et al*., 2000; Kronqvist *et al*., 2000; Gafvelin *et al*., 2001). For example, 87.8% (72 / 82) of asthmatic patients had positive skin prick tests to immunopurified native Der p 2 (Tsai *et al*., 2000) and 70%

(119 / 170) allergic patients in Venezuela had positive skin tests to recombinant Der p 2 (Lynch *et al*., 1997). This also showed that group 2 allergens are the major sensitizing allergens among mite-allergic subjects.

IgE binding activity for Pso o 2 was not determined. However, since *Psoroptes ovis* or the sheep scab mite, which is responsible for psoroptic scabies of cattle and sheep, rarely comes into contact with humans, the IgE binding activity of Pso o 2 is less relevant. Nonetheless, it shared around 40-54% amino acid sequence identity with other group II allergens (Temeyer *et al*., 2002).

2.4.1.3 **Group 3 allergens**

Der f 3 was purified and characterized by Heymann *et al*. (Heymann *et al*., 1989). Der f 3 was suggested to be a trypsin-like protein based on sequence comparison with Der p 3, a functionally characterized trypsin in *Der p* (Smith *et al*., 1996). It was reported that Der f 3 bound IgE in 16% (8 / 51) of the subjects tested (Heymann *et al*., 1989).

The first indication of the existence of Der p 3 was reported by Stewart *et al*. where they showed Der p 3 shared sequence similarity with trypsin and chymotrypsin from other organisms (Stewart *et al*., 1989). Native Der p 3 was later isolated from *Der p* extract using gel filtration and the cDNA of Der p 3 was subsequently identified as well (Smith *et al*., 1994). Der p 3 has a predicted molecular weight of 24985 daltons (Smith *et al*., 1994). Der p 3 was later proven functionally as a trypsin (Stewart *et al*., 1992).

Besides, Der s 3 was also identified in *Der s* (Ferrándiz *et al*., 1997). The purified Der s 3 has a molecular weight of 30000 daltons with 73% of reactivity in patients' sera tested (Ferrándiz *et al*., 1997). No further reports were available on Der s 3 in the literature.

Blo t 3 was reported recently separately by two groups. (Flores *et al*., 2003; Cheong *et al*., 2003 (b)). The full length Blo t 3 gene consists of 1138 base pairs. This includes 105 bp long 5' non-translated region and a open reading frame (ORF) from position 106-906 bp of the full length gene (Cheong *et al*., 2003 (b)). The IgE binding activity of Blo t 3 was rather weak although around 50% of the subjects selected reacted to it (Cheong *et al*., 2003 (b)).

Not much had been done on Eur m 3 except that its mRNA sequence had been submitted to Gene Bank and the IUIS database (Accession number: AF047615) (http://www.allergen.org/List.htm, Thomas *et al*., 2002).

2.4.1.4 **Group 4 allergens**

Group 4 allergen was first identified in *Der p* (Lake *et al.*, 1990) and *Eur m* (Mills *et al*., 1999). The cDNA of these allergens were subsequently identified by Mills *et al*. (Mills *et al*., 1999). Both Der p 4 and Eur m 4 genes coded for 496 amino acids and both sequences were 90% identical to each other (Mills *et al*., 1999). Both allergens had the same calculated molecular mass: around 57000 daltons (Table 2) though their recombinant forms migrated on SDS-PAGE at about 60000 daltons (Mills *et al*., 1999).

Native form Der p 4 had 46% IgE binding activity in mite-allergic adults and 25% in allergic children (Lake *et al*., 1990) whereas the recombinant form (His_6-tagged) had 30% (3 / 10) (Mills *et al*., 1999). However, none of the 10 mite-allergic patients in Mills *et al*.'s study responded to His_6-tagged recombinant Eur m 4 (Mills *et al*., 1999).

Blo t 4 had been identified as well (Dr. Cheong Nge, personal communication) but detailed information on the allergen remains to be published.

2.4.1.5 **Group 5 allergens**

In the literature, 3 group 5 allergens have been identified so far: Der p 5, Blo t 5 and Lep d 5 (Tovey *et al.*, 1989; Arruda *et al*., 1995; Eriksson *et al*., 2001; Table 2).

The molecular weight of mature Der p 5 allergen is 14000 (Tovey *et al*., 1989; Lin *et al*., 1994). 52% (13 / 25) of allergic subjects tested reacted positively to recombinant Der p 5 (expressed in *E. coli* system) in dot blot assay (Lin *et al*., 1994). A study in Venezuela also showed that 60% (102 / 170) allergic patients had positive responses in skin prick tests to recombinant Der p 5 (Lynch *et al*., 1997). There could be cross-reactivity between recombinant Der p 5 and recombinant Der p 7 (Lynch *et al*., 1997) but this requires more studies to confirm.

Group 5 allergen identified from *Blo t* had 43% sequence identity with Der p 5 (Arruda *et al*., 1995; Arruda *et al*., 1997). Blo t 5 is the only allergen identified so far from *Blo t* that is confirmed to be a major allergen. Various studies had showed the importance of this allergen (Arruda *et al*., 1995; Arruda *et al*., 1997; Kuo *et al*., 1999; Kuo *et al*., 2003; Manolio *et al*., 2003). To cite a few, Arruda *et al*. showed that 45-70% of *Blo t* allergic asthmatic patients had IgE to recombinant Blo t 5 (Arruda *et al*., 1995; Arruda *et al.*, 1997), Kuo *et al*. showed that 91.8% (134 / 146) and 73.5% (36 / 49) of asthmatic subjects from Taiwan and Malaysia were positive to recombinant Blo t 5 (Kuo *et al*., 2003) and in a larger cohort study, 261 subjects (46%) were sensitized to Blo t 5 (Manolio *et al*., 2003).

Lep d 5 was identified by Eriksson *et al*. using phage surface display technology (Eriksson *et al*., 2001). Recombinant Lep d 5 was recognized by 9% (4 / 45) *Lep d* positive subjects (Eriksson *et al*., 2001).

The function of group 5 allergens was unknown.

2.4.1.6 **Group 6 allergens**

Group 6 allergens are serine proteases and have chymotrypsin-like activity. Der p 6 and Der f 6 were first identified by Yasueda *et al*. in 1993 (Yasueda *et al*., 1993). Subsequently, Der p 6 and Der f 6 were separately cloned by Bennett *et al*. and Kawamoto *et al*. respectively (Bennett & Thomas, 1996; Kawamoto *et al*., 1999). Recombinant Der f 6 expressed in *E. coli* system was also reported (Kawamoto *et al*., 1999). Der f 6 showed 75.1% sequence identity with Der p 6 (Kawamoto *et al*., 1999).

Group 6 allergens in other mites have yet to be identified. Nevertheless, potential group 6 allergen from *Blo t* has been identified recently (Dr. Cheong Nge, personal communication, unpublished data).

2.4.1.7 **Group 7 allergens**

52% (88 / 170) of the allergic patients recruited in Lynch *et al*.'s study was positive to recombinant Der p 7 in skin prick tests (Lynch *et al*., 1997). Crossed RAST (radioallergosorbant assay) inhibition study between recombinant Der p 7 and recombinant Der p 5 suggested that there could be significant cross-reactivity between the two allergens (Lynch *et al*., 1997). Nonetheless, further studies were required to confirm this observation.

Group 7 from *Lep d* has also been identified and has been shown to bind IgE from 62% (28 / 45) *Lep d*-sensitized subjects (Eriksson *et al*., 2001).

2.4.1.8 **Group 8 allergens**

Group 8 allergen (Table 2) from *Der p* was identified by O'Neill and colleagues (O'Neill *et al*., 1994). It was known as Der p 15 when originally identified but later classified as Der p 8 (O'Neill *et al*., 1994). It showed 50% identity with Yb subunits of rat and mouse glutathione S-transferases class Mu (O'Neill *et al*., 1994).

77 / 193 (40%) of the mite extract sensitive subjects reacted to Der p 8 in IgE radioimmunoassays (O'Neill *et al*., 1994).

2.4.1.9 **Group 9 allergens**

Until now, only group 9 from *Der p* was identified and characterized (King *et al*., 1996; Table 2). Der p 9 has a molecular weight of 23780 daltons (King *et al*., 1996). Functionally, it is a serine protease that could cleave collagen (King *et al*., 1996). Der p 9 had a 92% IgE binding capacity in the 12 subjects selected in King *et al*.'s study (King *et al*., 1996). Nonetheless, larger cohort study is required to further confirm the high IgE binding capacity.

There was indication that the enzymatic function of Der p 9 could also trigger a non-allergic inflammatory response in the airways through the release of pro-inflammatory cytokines such as GM-CSF and eotaxin (Sun *et al*., 2001).

2.4.1.10 **Group 10 allergens**

Group 10 allergens are all tropomyosins (Aki *et al*., 1995; Asturias *et al*., 1998; Yi *et al*., 2002). Der f 10 (Mag44) consisted of 988 bp with an ORF of 284 amino acids and a molecular weight of 32954 daltons (Aki *et al*., 1995). Native Der f 10 bound IgE from 90.3% (28 / 31) of mite-sensitized asthmatic subjects (Aki *et al*., 1995). Nonetheless, Der p 10, though another tropomyosins from mites, had only very low IgE binding frequency of 5.6% only (Asturias *et al*., 1998).

Yi *et al*. identified Blo t 10 cDNA from *Blo t* cDNA library recently using mouse anti-Der p 10 antibodies (Yi *et al*., 2002). The sequence was subsequently expressed in *E. coli* as a GST-fusion protein and the recombinant Blo t 10 had a molecular weight of around 35000 daltons in 7.5% SDS-PAGE after being cleaved from GST (Yi *et al*., 2002). The recombinant Blo t 10 had 20% (7 / 35) IgE-binding

frequency in skin prick tests (Yi *et al*., 2002). Lep d 10 has recently been cloned and characterized by Saarne *et al*. (Saarne *et al*., 2003). It consisted of 284 amino acids with a calculated molecular mass of 32930 daltons. Lep d 10 showed 96% sequence identity with Der f 10 and Der p 10. His_6-tagged Lep d 10 had a molecular weight of 33775 daltons as determined by mass spectrometry though its calculated molecular mass was 33752 daltons. Recombinant Lep d 10 has 13% IgE-binding frequencies (Saarne *et al*., 2003).

2.4.1.11 **Group 11 allergens**

Group 11 allergens are large proteins compared to allergens from group 1 to 10 (Table 2). Group 11 allergen had a molecular weight of 98000 daltons (Tsai *et al*., 1998 (b); Ramos *et al*., 2001). Group 11 allergen in *Der f* was identified by Tsai *et al*. while the *Blo t* version of it was identified by Ramos *et al*. (Tsai *et al*., 1998 (b); Ramos *et al*., 2001). Functionally, group 11 allergens are paramyosins based on sequence identity with paramyosins from other organisms (Tsai *et al*., 1998 (b); Ramos *et al*., 2001).

Group 11 allergens had significant IgE binding capacity (Tsai *et al*., 1998 (b); Ramos *et al*., 2001). 87.5% (21 / 24) of mite-sensitized patients were positive to Der f 11 in skin prick tests while 52% (33 / 63) of *Blo t*-sensitized subjects had IgE towards recombinant Blo t 11 (Ramos *et al*., 2001).

2.4.1.12 **Group 12 allergens**

Group 12 was only identified in *Blo t* until now (Puerta *et al*., 1996 (b)). Based on sequence analysis, Blo t 12 should have a mature protein of molecular weight 14206 and a plausible 20 amino acids signal peptide (Puerta *et al*., 1996 (b)). The function of the allergen was unknown but it was possible it had chitin-binding or

chitinase activity based on the fact that it had a chitin-binding domain (Stewart & Robinson, 2003). Blo t 12 had a 50% (16 / 32) IgE binding frequency in allergic asthmatics (Puerta *et al*., 1996 (b)). However, the prevalence and the importance of sensitization to Blo t 12 in the general population remain to be shown.

2.4.1.13 **Group 13 allergens**

Three group 13 allergens had been reported in the literature: Blo t 13, Aca s 13, Lep d 13 (Caraballo *et al*., 1997; Eriksson *et al*., 1999; Eriksson *et al*., 2001). Blo t 13, an allergen similar to fatty acid binding proteins, was cloned and characterized by Caraballo *et al*. (Caraballo *et al*., 1997). Subsequently, group 13 allergens in *Aca s* and *Lep d* were identified by Eriksson *et al*. (Eriksson *et al*., 1999; Eriksson *et al*., 2001).

In terms of IgE binding capacity, 11% (5 / 45) of allergic subjects reacted to Blo t 13 using RAST (Caraballo *et al*., 1997); 23% (3 / 13) of the subjects in Eriksson *et al*.'s study were positive to Aca s 13 (Eriksson *et al*., 1999); 13% (6 / 45) of *Lep d*-sensitized subjects had IgE against recombinant Lep 13 (Eriksson *et al*., 2001).

2.4.1.14 **Group 14 allergens**

Group 14 allergens are another group of high-molecular weight allergens (Fujikawa *et al*., 1996; Epton *et al*., 1999). Der f 14 (originally known as Mag 3 when first identified and Eur m 14 have a molecular weight of 177000 daltons (Fujikawa *et al*., 1996; Epton *et al*., 1999). Both the native and the recombinant form of Der f 14 could bind IgE: 70% (16 / 23) and 39% (9 / 23) of mite-sensitized asthmatic patients respectively (Fujikawa *et al*., 1996). Der f 14 and Eur m 14 had strong similarity with insect apolipophorins indicating similar function (Epton *et al*., 1999).

There was, however, insufficient data on Der p 14 in the literature except that it was listed in the IUIS database. (http://www.allergen.org/List.htm). Only partial sequences of Der p 14 had been described thus far (Epton *et al*., 1999).

2.4.1.15 **Group 15 allergens**

Group 15 allergen was only identified in *Der f* (McCall *et al*., 2001). It was one of the few allergens that were identified to be important in canine allergy (McCall *et al*., 2001). Der f 15, which has a predicted molecular weight of 61108 daltons, is considered as a high molecular weight allergen because it is highly glycosylated, giving it an actual molecular weight of 98000 daltons and 109000 daltons (McCall *et al*., 2001). Sequence analysis suggested that Der f 15 is a chitinase (McCall *et al*., 2001). As for the allergenicity of Der f 15, 92.6% (25 / 27) *Der f* extract-allergic dogs in McCall *et al*.'s study had IgE against Der f 15 (McCall *et al*., 2001). The importance of Der f 15 in human allergic subjects remains to be studied.

2.4.1.16 **Group 16**

Der f 16, a gelsolin, reported by Kawamoto *et al*, has a predicted molecular weight of 55131 daltons (Kawamoto *et al*., 2002 (b)). The recombinant Der f 16, with the same molecular weight, had variable IgE binding frequencies among asthmatic subjects, depending on the cohort chosen: 17.9% (5 / 28) in one group of subjects and 47% (8 / 17) in another (Kawamoto *et al*., 2002 (b)).

2.4.1.17 **Group 17 to 19 allergens**

Der f 17, according to IUIS database, was a calcium binding EF protein, with a molecular weight of around 53000 daltons (http://www.allergen.org/List.htm, Thomas *et al*., 2002). Other than this, there was inadequate information in the literature on this allergen.

Der f 18, an allergen homologous to chitinase, was identified in *Der f* by Weber *et al*. (Weber et al., 2003). It consisted of 462 amino acids, including a 25 amino acid signal sequence and a 437 amino acids mature protein sequence. The calculated molecular mass for Der f 18 was 50000 daltons. This was also one of the few allergens to have been shown to be allergenic to both humans and dogs (Weber *et al*., 2003). According to Weber *et al*., 54% of the *Der f* sensitized patients reacted to Der f 18 whereas 57-77% of *Der f* sensitized dogs did the same (Weber *et al*., 2003).

A putative anti-microbial peptide was identified by Cheong and co-workers. (reported in this study) and was named BtA2 originally (Lim *et al*., 2002). It was later classified as Group 19 by the IUIS Allergen Nomenclature sub-committee.

2.4.1.18 **Undesignated allergens**

Besides the 19 designated groups of allergens, there are some domestic mite allergens in the literature that had not been grouped (Table 2) (Aki *et al*., 1994; Saarne *et al*., 2003). The first one being a putative member heat shock protein (hsp) 70 family was identified by Aki *et al*. in *Der f* (Aki *et al*., 1994). The second one was a α-tubulin from *Lep d* (Saarne *et al*., 2003).

2.5 *Monoclonal antibodies in mite allergen studies*

The hybridoma technology was first demonstrated by Köhler and Milstein in 1975 to be useful in the production of monoclonal antibody against antigen of interest (Köhler & Milstein, 1975). This technology involves the fusion of spleen cells (antibody producing cells (B-cells)) and myeloma cells (immortalized cells), thus generating a hybridoma which could produce antibody and at the same time immortalized (Köhler & Milstein, 1975). Since then, this technology has expanded and

became a valuable tool to generate useful reagents in everyday molecular and cell biology research.

2.5.1 Applications of monoclonal antibodies in allergy studies

Generally, monoclonal antibodies are used in identification and purification of allergens, epitope mapping studies and standardization of mite allergen extracts (Chapman *et al*., 1987; Luczynska *et al*., 1989; Härfast *et al*., 1992; Yunginger & Adolphson, 1992; Ovsyannikova *et al*., 1994; Ferrándiz *et al*., 1995; Shen *et al*., 1995; Shen *et al*., 1996; Ferrándiz *et al*., 1997; Peng *et al*., 1998; Tsai *et al*., 2000; Labrada *et al*., 2002; Park *et al*., 2002; Parvaneh *et al*., 2002; Trombone *et al*., 2002; Ramos *et al*., 2003). A number of native allergens (Der p 1; Der p 2; Blo t 11) have been immunopurified and characterized (Chapman *et al*., 1987; Tsai *et al*., 2000; Ramos *et al*., 2003). Monoclonal antibodies have also been used to identify allergens from the same group of allergen, for example, Eur m 2 identified by monoclonal antibody against Der p 2; Blo t 11 immunopurified using monoclonal antibody against Der f 11 (Morgan *et al*., 1997; Ramos *et al*., 2003).

ELISA-based assays developed using monoclonal antibodies for Der p1, Der f 1, Der p 2, Lep d 2 and Der p 7 were useful in various studies: 1.) to quantify the level Der p 1, Der f 1, Der p 2 and Lep d 2 from dust samples (Luczynska *et al*., 1989; Ovsyannikova *et al*., 1994; Parvaneh *et al*., 2002); 2.) to detect Der f 1-specific IgE (Peng *et al*., 1998); 3.) to study the allergenicity differences of Der p 2 isoallergens (Park *et al*., 2002); 4.) epitope mapping of Der p 1, Der f 1, Der p 2 and Der p 7 (Chapman *et al*., 1984; Chapman *et al*., 1987; Ovsyannikova *et al*., 1994; Shen *et al*., 1996); 5.) to quantify Der p 1 and Der p 2 specific IgE (Trombone *et al*., 2002); 6.) to standardize commercial mite allergen extracts (Ovsyannikova *et al*., 1994).

2.5.2 Methods in monoclonal antibody productions

There are basically two major protocols in producing monoclonal antibodies. The first one was the more traditional and widely adopted method where it involved immunization of mice and fusion of mice spleen cells with myeloma cells to generate monoclonal antibody producing hybridomas while the second was relatively new which was derived from phage display technology (Köhler & Milstein, 1975; Smith, 1985; McCafferty *et al*., 1990; Clackson *et al*., 1991; Marks *et al*., 1991).

The widely adopted protocol for monoclonal antibody generation has not changed significantly since its introduction in 1975 in that it still involves the fusion between myeloma cells and B cells to generate antibody-producing hybridomas (Köhler & Milstein, 1975; Spitz *et al*., 1984; Svalander *et al*., 1987; Hong *et al*., 1989; Quak *et al*., 1990; Nilsson & Larsson, 1990; Barry *et al*., 1994; Ulivieri *et al*., 1996; Kilpatrick *et al*., 1997; Peet *et al*., 1997; Rizzuto *et al*., 1999; Tearina Chu *et al*., 2000; Kilpatrick *et al*., 2000; Velikovsky *et al*., 2000; Kasinrerk *et al*., 2002; Velikovsky *et al*., 2002; Ramos *et al*., 2003; Yang *et al*., 2003). Nonetheless, various immunization protocols have been introduced. These methods not only differ in the forms of antigen used but also in the routes of administration (Spitz *et al*., 1984; Svalander *et al*., 1987; Hong *et al*., 1989; Quak *et al*., 1990; Nilsson & Larsson, 1990; Barry *et al*., 1994; Ulivieri *et al*., 1996; Kilpatrick *et al*., 1997; Peet *et al*., 1997; Rizzuto *et al*., 1999; Tearina Chu *et al*., 2000; Kilpatrick *et al*., 2000; Velikovsky *et al*., 2000; Kasinrerk *et al*., 2002; Velikovsky *et al*., 2002; Ramos *et al*., 2003; Yang *et al*., 2003).

In terms of the antigens used for immunization, aside from the traditional immunization using protein with complete Freund's or incomplete Freund's adjuvants, or with alum (Quak *et al*., 1990; Peet *et al*., 1997; Ulmer *et al*., 2000; Wang *et al*., 2000), DNA immunization employing plasmid DNA expressing the gene of the protein

of interest has also been reported (Barry *et al*., 1994; Attanasio *et al*., 1997; Tearina Chu *et al*., 2000; Kilpatrick *et al*., 2000; Velikovsky *et al*., 2000; Ramos *et al*., 2003; Wolfowicz *et al*., 2003; Yang *et al*., 2003). The incorporation of electroporation into DNA immunization protocol that involves intramuscular injection of plasmid DNA has been shown to greatly enhance the immune responses (Rizzuto *et al*., 1999; Widera *et al*., 2000). This was because electroporation could increase the transfection frequencies (Aihara & Miyazaki, 1998; Mir *et al*., 1999; Widera *et al*., 2000). The advantage of DNA immunization is that it enables investigators to induce antibody response in animals without the need to purify or generate antigen that could sometimes be difficult to produce or isolate (Tang *et al*., 1992; Costagliola *et al*., 1998; Moonsom *et al*., 2001; Yang *et al*. 2003). DNA immunization could also induce antibody responses when conventional immunization protocol had failed (Hong *et al*., 1989). On top of that, DNA immunization could also induce more antibodies that recognize native epitopes compared to immunization using recombinant protein (Attanasio *et al*., 1997; Costagliola *et al*., 1998). Nonetheless, the success of genetic immunization also depended on various other factors such as the nature of the antigen, mouse strain and the presence or absence of CpG motifs (Berzofsky *et al*., 1977; Lee & Sung, 1998; Chatel *et al*., 2003).

Besides that, the delivery routes of antigens also affect the immune response (Boyle *et al*., 1997; Kasinrerk *et al*., 2002). Various routes of delivery had been reported, namely subcutaneous, intradermal, intraperitoneal, intramuscular and intrasplenic, with various degrees of success in inducing immune responses (Spitz *et al*., 1984; Hong *et al*., 1989; Boyle *et al*., 1997; Kasinrerk *et al*., 2002)

The phage display method totally bypasses the immunization and fusion processes (Clackson *et al*., 1991; Marks *et al*., 1991). Basically, bacteriophage such as

fd bacteriophage was modified to express antibody fragments on its coat and those phages that express antibody fragments that bind to antigen of interest could be enriched and selected through a process known as "biopanning" (McCafferty *et al*., 1990; Clackson *et al*., 1991; Smothers *et al*., 2002).

2.6 *Antimicrobial peptides (AMPs): a brief introduction*

Antimicrobial peptides (AMPs) had been discovered in organisms from almost all kingdoms: from mammals, plants, to microorganisms (Papagianni, 2003). As their name suggests, AMPs could kill a broad range of microorganisms, which includes bacteria (both gram-positive and negative), fungi and even certain viruses (Gallo *et al*., 2002). AMPs are important components of innate immune response as they offered the first-line defense against infections (Hoffmann *et al*., 1996; Gallo *et al*., 2002).

All AMPs share a few common features: 1.) low molecular weight (below 5000); 2.) a positive net charge at physiological pH; 3.) most had amphiphilic α-helices or hairpin-like β-sheets or a mixed of the two (Bulet *et al*., 1999). The most common and potent AMPs known to date could be classified as cationic peptides (Vizioli & Salzet, 2002). Cationic peptides could be further classified into 3 classes: 1.) linear peptides forming α-helices without cysteine residues; 2.) cyclic peptides with cysteine residues; 3.) peptides containing excessive proline and / or glycine residues (Dimarcq *et al*., 1998; Bulet *et al*., 1999; Vizioli & Salzet, 2002; Papagianni, 2003). Other groups of AMPs, classified respectively as anionic peptides, aromatic dipeptides and oxygen-binding proteins, in general, have weak anti-microbial properties and their importance remains to be elucidated (Vizioli & Salzet, 2002).

Linear α-helix peptides		
Peptide	**Source**	**Antimicrobial activity**
Cecropins	Insects, pig	Bacteria, fungi, virus, protozoa, metazoa
Clavanin, styelin	Tunicates	Bacteria
Buforins	Amphibians	Bacteria, protozoa
Cyclic peptides with cysteine residues		
α-Defensins	Humans, rabbits	Bacteria, fungi, virus
β-Defensins	Cattle	Bacteria, fungi, virus
Insect defensins	Insects	Gram positive bacteria
Ascaris suum antibacterial factor (ASABF)	Nematode	Bacteria, yeast
Myticin	Mussels	Bacteria
Mytilin B	Mussels	Bacteria
MGD-1	Mussels	Bacteria
MGD-2	Mussels	Bacteria
Peptides rich with certain amino acid		
Drosocin	Fruit fly	Bacteria
Metalnikowin	Hemipteran	Bacteria, fungi
Bac-7	Cattle, sheep	Bacteria

Table 3: Examples of AMPs classified as cationic peptides. Examples from each different sub-groups (adapted from Papagianni, 2003; Vizioli & Salzet, 2002; Mitta *et al*., 2000; Mitta *et al*., 1999; Zhang *et al*., 2000; Kato & Komatsu., 1996).

AMPs are produced in different manner in different organisms. Some are constitutively expressed, others inducible while the remaining uses both mechanisms (Lehrer & Ganz, 1999).

Chapter 3

3 Mite sensitization profile study

3.1 *Mite sensitization in South East Asia*

Countries in South East Asia are characterized by their tropical to subtropical climate with high humidity throughout the year. These climate conditions are especially suitable for mite growth (Arlian *et al*., 1998 (a-b); Chew *et al*., 1999 (b)). In fact, various studies in South East Asia had shown the high concentration of domestic mites (*Blo t* (predominant in Singapore), *Der p* (predominant in Thailand and Malaysia) in the environment (Ho, 1986; Malainual *et al*., 1995; Chew *et al*., 1999). It was also known that exposure to high concentration of mite allergen (≥2 μg / g of dust) increased the potential of sensitization in atopic individuals (Lau *et al*., 1989). In addition, the positive association of mite sensitization to allergic diseases had been shown (Rizzo *et al*., 1993; Stanaland *et al*., 1994; Ho *et al*., 1995; Droste *et al*., 1996; Ferrándiz *et al*., 1996; Nelson *et al*., 1996; Boulet *et al.*, 1997; Leung *et al*., 1997; Tsai *et al*., 1998 (a); Baratawidjaja *et al*., 1999; Mori *et al*., 2001; Verini *et al*., 2001). Therefore, it is important to study the prevalence of mite sensitization in South East Asia using a panel of individual recombinant mite allergens.

The prevalence of mite sensitization among allergic patients in South East Asia was high, ranging from 70-96% among the subjects studied (Woodcock & Cunnington, 1980; Ho *et al*., 1995; Baratawidjaja *et al*., 1999; Chew *et al*., 1999 (a); Kuo *et al*., 1999). The majority of the subjects in these studies were adolescents and adults who were suffering from allergic rhinitis and / or asthma (Woodcock & Cunnington, 1980; Ho *et al*., 1995; Baratawidjaja *et al*., 1999; Chew *et al*., 1999 (a); Kuo *et al*., 1999).

For instance, a study conducted by Ho *et al*. in Malaysia demonstrated that 82% (257 / 314) of the allergic rhinitis subjects were sensitized to *Der p* and *Der f* respectively (Ho *et al*., 1995).

Most of the studies reported in South East Asia to date gave data on the prevalence of mite sensitization among allergic disease patients only (Woodcock & Cunnington, 1980; Ho *et al*., 1995; Baratawidjaja *et al*., 1999; Chew *et al*., 1999 (a); Kuo *et al*., 1999; Trakultivakorn & Nuglor, 2002). There was only one study on the prevalence of mite sensitization among the general population where it was demonstrated that 60.2% (193 / 321) of the randomly selected school children in Kota Kinabalu (situated in Sabah, Malaysia) were sensitized to *Der p* (Leung *et al*., 1997). This prevalence frequency was unusually high as compared to the study in Europe where the prevalence of mite sensitization ranged from 6.7% to 35.1% (Burney *et al*., 1997).

All the studies so far showed that mite sensitization is an important factor among allergic rhinitis and / or asthma patients (Woodcock & Cunnington, 1980; Ho *et al*., 1995; Baratawidjaja *et al*., 1999; Chew *et al*., 1999 (a); Kuo *et al*., 1999; Trakultivakorn & Nuglor, 2002). In order to enable better treatment, it is important to pin point the major sensitizing mite allergens among allergic rhinitis / asthma patients. This could only be done using recombinant mite allergens.

3.2 *Significance of the study*

First of all, this study was aimed at providing more information on the frequency of mite sensitization among allergic disease sufferers in South East Asia. Although there were a number of studies on the prevalence of mite sensitization in South East Asia (Woodcock & Cunnington, 1980; Ho *et al*., 1995; Leung *et al*., 1997; Baratawidjaja *et al*., 1999; Chew *et al*., 1999 (a), mite crude extracts were used as the

reagents. The usefulness of recombinant and purified domestic mite allergens as reagents for sensitization studies had not been fully evaluated in populations in this region of the world.

Three recombinant and purified domestic mite allergens (Der p 1, Der p 2 and Blo t 5) had been shown to be important in both Singapore and Taiwan (Kuo *et al*., 1999). Interestingly, Blo t 5 appeared to be more important among Singaporean subjects compared to Taiwanese subjects (Kuo *et al*., 1999). Nevertheless, the number of Singaporean subjects that were tested for their specific IgE against the three allergens was small in the study. More studies using larger cohorts were needed to further confirm these findings because Trakultivakorn & Nuglor demonstrated that Der p 1 and Der p 2 and not Blo t 5 were important in Thailand. It was also important to know whether these three allergens could potentially replace the use of mite crude extract.

Therefore, in this study, the sensitization profile of rhinitis and asthma patients against mite crude extracts from *Blo t* and *Der p* and recombinant allergens Der p 1, Der p 2 and Blo t 5 were performed. This is to further compare with the findings by Kuo *et al*. (Kuo *et al*., 1999). At the same time, the usefulness of recombinant and purified domestic mite allergens sensitization study would be evaluated.

3.3 *Materials and methods*

The water used in these methods was Milli-Q water unless otherwise specified.

3.3.1 Allergens

3.3.1.1 *Blo t* and *Der p* mite crude extract

Blo t and *Der p* mite crude extracts were prepared as described previously (Kuo *et al.*, 1999). Briefly, 5 g of frozen dust mites (*Blo t*, grown in-house as described

previously (Yi *et al*., 1999) or lyophilized *Der p* (Commonwealth Serum Laboratories, Parkville, Australia) was homogenized in the presence of liquid nitrogen using mortar and pestle. Protein extraction was performed using PBS (phosphate-buffered saline) (for sensitization study) or TBS (Tris-buffered saline) (for purification of native protein), both containing 2 mM phenylmethyl-sulfonyl fluoride (PMSF) (Sigma, St. Louis, MO, USA) and 1 mM ethylenediamine tetraacetic acid (EDTA), pH 8.0 (Bio-Rad, Hercules, CA, USA). The mixture was centrifuged at 15000 x g for 15 minutes and the supernatant was dialyzed overnight at 4 °C against 1X PBS. Protein concentration was determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). The protein extracts were stored in aliquots at -80 °C.

3.3.1.2 **Preparation of Der p 1, Der p 2 and Blo t 5 allergens**

Native Der p 1 was purified from spent mite medium by affinity chromatography, using the monoclonal antibody 4C1 (Chapman *et al*., 1987). Recombinant Der p 2 (Chua *et al*., 1990; Chua *et al*., 1996) and Blo t 5 (Arruda *et al*., 1997) were produced in the *Pichia pastoris* yeast expression system and purified using chromatographic methods (Kuo *et al.*, 1999; Goh *et al.*, 2001).

3.3.2 Selection of subjects

A total of 229 sera were obtained from randomly selected adult volunteers who attended a national rhinitis survey study in the ear, nose and throat outpatient clinic of the National University Hospital, Singapore. Out of this total, 124 subjects (62 males and 62 females, aged 7 - 73 years; Mean age of 34 years), were diagnosed with persistent rhinitis by clinician (Bousquet *et al*., 2001). The definition of rhinitis was based on the ICR algorithm recommendation for treatment of rhinitis (Lund *et al*., 1994). In addition, 105 subjects (59 males and 46 females; aged 7 – 73 years; Mean

age: 38 years old) with no history of rhinitis were recruited for comparison. Approval to conduct this study was granted by the National Medical Research Council (NMRC) of Singapore and the institutional review board of the Medical Faculty, National University of Singapore.

A total of 153 sera (94 sera from adult asthmatics (age mean age 46.4 years old); 49 sera from children asthmatics) visiting the asthmatic clinic in University Hospital, University of Malaya, Malaysia, were also included. All the patients did not have chronic obstructive pulmonary disease (COPD) as diagnosed by clinician.

3.3.3 ELISA for detection of sensitization profile

Specific IgE in the sera against Blo t 5, Der p 1 and Der p 2 were determined using enzyme-linked immunosorbent assay (ELISA) as previously described (Kuo *et al*). Briefly, 250 ng of allergen was coated in each well of the 96-well microtitre plate (Corning, NY, USA) overnight in 0.1 M $NaHCO_3$ (Sigma, St. Louis, MO, USA). The plate was then washed 3 X with PBS-Tween 20 (0.05%, v/v) (washing solution) using Columbus washer (TECAN, Grödig, Austria). All subsequent washing steps were performed in the similar way. The plate was then blocked with washing solution containing 1% (w/v) BSA (Sigma, St. Louis, MO, USA) (blocking and dilution solution) for two hours. Then, the plate was washed. Sera diluted 1 : 5 in dilution solution was dispensed 50 µl / well and incubated overnight. The next day, the plate was washed and incubated with mouse anti-human IgE biotin conjugated (Southern Biotechnology, Alabama, USA) (1 : 2000 dilution) for 1 hour at room temperature. Again, the plate was washed and incubated with ExtrAvidin® alkaline phosphatase conjugate (Sigma, St. Louis, MO, USA) (1 : 2000 dilution) at room temperature for 1 hr. The plate was washed and the substrate, β-Nitrophenyl phosphate (Sigma, St. Louis, MO, USA), was then added. Optical density (OD_{405nm}) readings were obtained using

Shell Rainbow Reader (TECAN, Grödig, Austria) at 30 minutes and 1 hour after the addition of substrate.

Control sera for Singapore subjects were from a set of sera that showed negative results in ELISA against the allergens tested whereas control sera for Malaysia asthmatic subjects were from patients who gave no observable wheals to all the allergens in the SPT. The cut off value for individual allergen ELISA read out was based on the mean of all the cut offs for individual allergens of which were determined using the mean plus two standard deviations of these control sera against respective allergen. However, cut offs for mite crude extracts (*Der p* extract and *Blo t* extract) were determined independently from individual allergens. Their cut offs were determined using the mean plus two standard deviations of the ELISA readings of control sera to the respective extract.

3.3.4 Skin Prick Tests (SPT)

Skin prick tests (SPT) was performed as described previously (Chew *et al*., 1999 (b)) on all the 94 Malaysian adult asthmatics. The wheal size induced by applying total extract of *Blo t* / *Der p* extract (Greer Laboratories Inc., Lenoir, NC, USA), allergens Blo t 5, Der p 1 or Der p 2 on the forearm (pricking site) was considered positive if its diameter was larger than 3 mm after 30 minutes (Chew *et al*., 1999 (b)). Histamine (1 mg / ml) and PBS were used as positive and negative control respectively. All subjects were requested not to take antihistamines for 24 hours before SPT was performed.

3.3.5 Computer-aided statistical analysis

The OD_{405nm} readings from Singapore subjects were analyzed using Mann Whitney statistical test (SPSS for Windows Ver. 10.0.5). Mann Whitney statistical test

(non-parametric methods) was chosen because it required fewer assumptions on the population where the data was derived. Correlation coefficients and odds ration analysis were calculated using Excel (Microsoft® Excel 2002 (10.3506.3501)SP-1).

3.4 *Results*

3.4.1 Sensitization profile of Singapore subjects

In this study we have determined the dust mite allergens sensitization profile for four subgroups of subjects: rhinitis and non-rhinitis subjects from Singapore; adults and children with asthma from Malaysia.

The sensitization profile of rhinitis subjects to the domestic mite allergens used in this study was as follow: *Blo t* extract +: 91 / 124 (73%); Blo t 5 +: 62 / 124 (50%); *Der p* extract +: 61 / 124 (49%); Der p 1 +: 53 / 124 (43%); Der p 2 +: 45 / 124 (36%). The non-rhinitis subjects' sensitization profile was as follows: *Blo t* extract +: 60 / 105 (57%); Blo t 5 +: 24 / 105 (23%); *Der p* extract +: 38 / 105 (36%); Der p 1 +: 14 / 105(13%); Der p 2 +: 17 / 105 (16%).

There were significant differences in the levels of allergen specific IgE in rhinitis versus non-rhinitis subjects. In fact, the rhinitis subjects generally had a higher titre of IgE (inferring from higher OD reading) than non-rhinitis subjects (Figure 3). In addition, a higher number of rhinitis subjects were sensitized to each of the allergens compared to non-rhinitis group. In the rhinitis cohort, 81 / 124 (65%) reacted to at least one allergen from *Blo t* and / or *Der p* (Blo t 5 / Der p 1 / Der p 2), while in the non-rhinitis individuals, the corresponding figure was 36 / 105 (34%) (Figure 4).

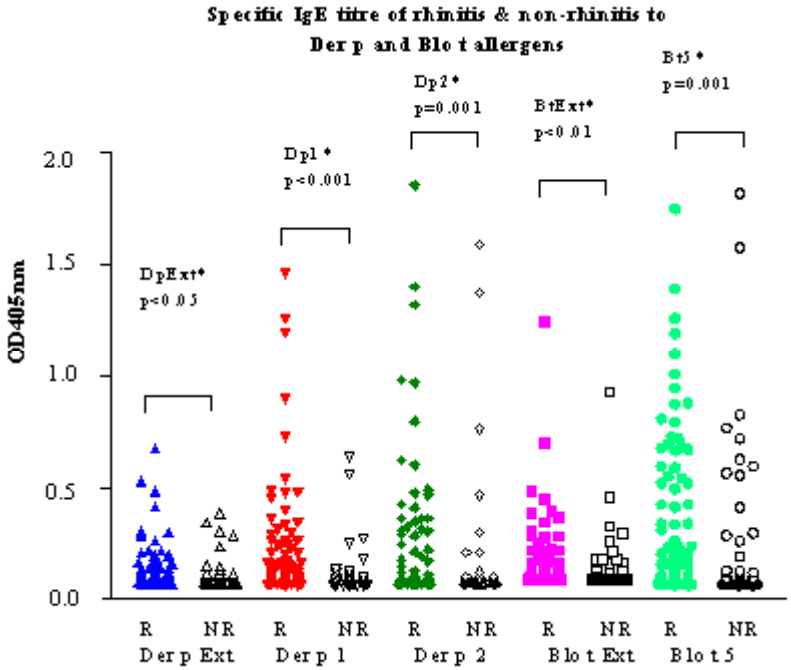

Figure 3: Allergen specific IgE titre from rhinitis (R) (n=124) and non-rhinitis (NR) (n=105) subjects from Singapore. Significant differences were observed between the two groups for all allergens when analyzed using Mann Whitney statistical analyses. "*" means significant difference was observed. Ext = Extract; Bt = Blo t; Dp = Der p. Cut off = 0.093 for Der p 1, Der p 2, Blo t 5; cut off = 0.101 for *Blo t* extract; 0.090 for *Der p* extract.

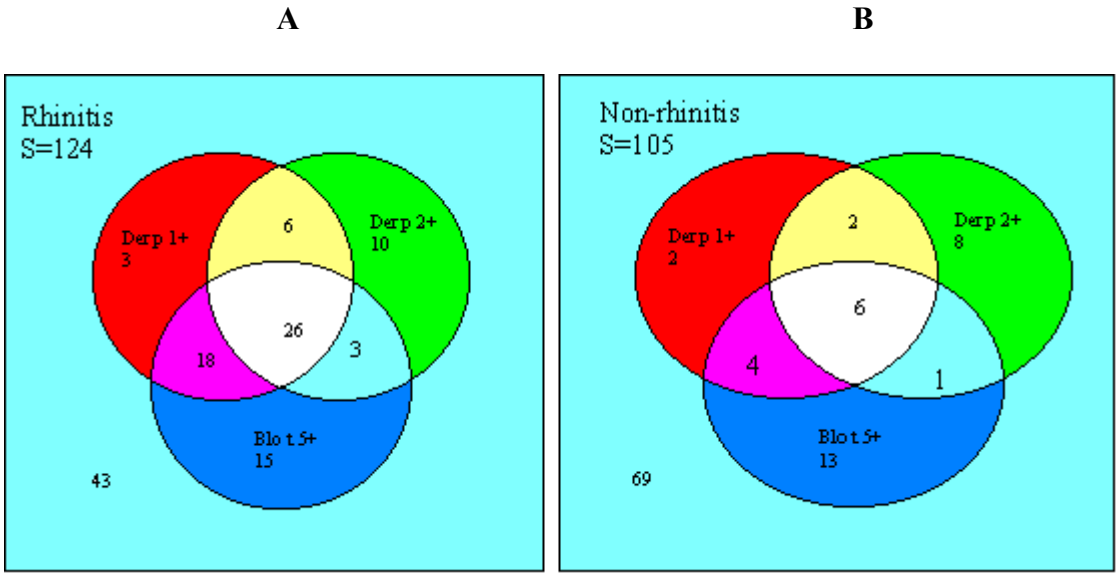

Figure 4: Sensitization profile of Singapore rhinitis (A) and non-rhinitis (B) subjects based on ELISA results. Cut off = 0.093 for all allergens in both diagram A and B.

Correlation between the single allergens among rhinitis subjects was: Der p 1 – Der p 2: r = 0.60; Blo t 5 – Der p 1: r = 0.64; Blo t 5 – Der p 2: r = 0.78. Correlation for the same parameters for non-rhinitis: Der p 1- Der p 2: r = 0.71; Blo t 5 – Der p 1: r = 0.51; Blo t 5 – Der p 2: r = 0.45.

	Odds Ratio (95% confidence interval)
***Blo t* Extract**	2.1 (1.2, 3.6)
Blo t 5	3.4 (1.9, 6.0)
***Der p* Extract**	1.7 (1.0, 2.9)
Der p 1	4.9 (2.5, 9.4)
Der p 2	2.9 (1.6, 5.6)

Table 4: The association of sensitization to various domestic mite allergens with rhinitis patients.

	Odds Ratio (95% confidence interval)
Mite sensitization (MS)	2.2 (1.1, 4.3)
MS (Der p 1, Der p 2, Blo t 5)	3.6 (2.1, 6.2)
MS (extracts alone)	1.9 (1.1, 3.5)

Table 5: The association of overall mite sensitization to rhinitis.

As shown in Table 4, all the allergens used were associated with rhinitis. This indicated that for example, if one was sensitized to Blo t 5, he / she had 3.4 times higher chance of having rhinits (Table 4). All allergens except for *Der p* extract, generally were associated with increase chance of suffering from rhinitis, judging from the 95% confidence interval (Table 4). The associations of Der p 1 and Blo t 5 sensitizations to rhinitis were the strongest (Table 4). Taken together, mite sensitization status determined solely using Der p 1, Der p 2 and Blo t 5 had higher association with rhinitis (Table 5).

3.4.2 Sensitization profile of Malaysian patients with asthma

Our study on Malaysian adult with asthma showed that 39% of the adult patients with asthma were sensitized to Der p 1; 32% to Der p 2; 37% to Blo t 5 based on ELISA results. The corresponding sensitization profiles in the asthmatic children were 57% to Der p 1, 39% to Der p 2 and 90% to Blo t 5 (Figure 5). Due to the fact that the number of subjects in both the adult and the children cohorts were relatively small, no statistical tests were performed to compare the significance of the differences between the two groups. SPT results coincided quite well with ELISA results, as shown by the few individuals that were detected only by either tests but not both (Table 6).

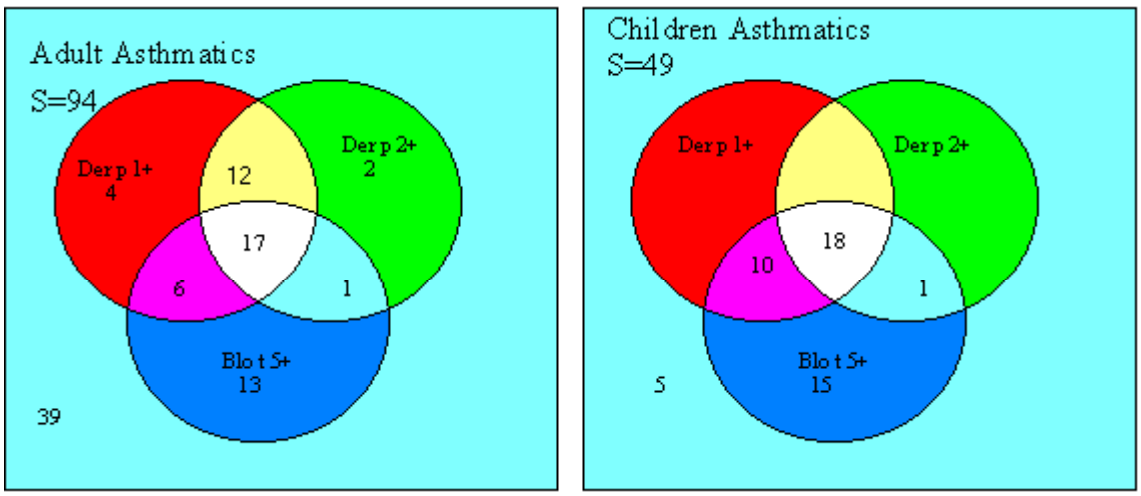

Figure 5: **Sensitization profile of adult (A) and children patients with asthma (B) from Malaysia based on ELISA results. Cut off = 0.15 for all allergens in both diagram A and B.**

N=94	Der p 1	Der p 2	Blo t 5
SPT +	33 (35%)	32 (34%)	30 (32%)
ELISA +	39 (41%)	32 (34%)	37 (39%)
ELISA +; SPT +	24 (26%)	25 (26%)	27 (29%)

Table 6: Comparison of Malaysia asthmatic adults' skin prick tests (SPT) and ELISA results from Malaysian adults with asthma.

Considering SPT results alone, 36 / 94 (38%) of the Malaysian adults with asthma were positive to *Der p* extract while 32 / 94 (34%) were positive to *Blo t* extract, both of which were quite similar to the results obtained from using individual allergens (Table 6). 18 / 36 (50%) of the *Der p* extract positive subjects were also positive to Der p 1 and Der p 2 SPT. In addition, 6 / 36 (17%) were positive to Der p 1, 4 / 36 (11%) were positive to Der p 2. In other words, 28 / 36 (78%) *Der p* extract (SPT) positive subjects could be accounted for by combining Der p 1 and Der p 2 SPT. On the other hand, Blo t 5 SPT managed to detect 22 / 32 (69%) of the *Blo t* extract positive (SPT) subjects.

Besides, the correlation between Der p 1 and Der p 2 sensitization was 0.77 for adult asthmatics, 0.94 for children. There was less correlation between Blo t 5 sensitization and either Der p 1 or Der p 2 (Blo t 5 – Der p 1 (adult) = 0.47, (children) = 0.22; Blo t 5 – Der p 2 (adult) = 0.41, (children) = 0.27). The slightly higher correlation between Blo t 5 and Der p 2 could be due to the possibility that both mite allergens were important early sensitizing agents.

The other interesting observation in this study was the frequency of multi-allergen sensitization in the asthma and rhinitis populations (Figure 4; Figure 5). However, certain individual allergen had higher frequency of sensitization than other. For instance, a majority of the patients were sensitized to Blo t 5. This was true for patients from Singapore and Malaysia.

3.5 *Discussion*

3.5.1 Sensitization profile of Singapore subjects

Our study on Singaporean subjects showed that a high percentage of the rhinitis patients were sensitized by both *Der p* and *Blo t* mites. These subjects showed sensitivity to at least one major allergen from *Blo t* and *Der p* mites (Figure 4). Our results also revealed that 34% of the non-rhinitis subjects were also sensitized by domestic mite allergens (Figure 4). The data suggest that mite sensitization in our general population is highly prevalent and that allergen sensitization does not always lead to clinical symptoms implying that the relevance of serum IgE to disease prediction remains controversial (Burrows *et al*., 1989; Smith, 1992; Hogan *et al*., 1997; Lynch *et al*., 1998; Niederberger *et al*., 2001). For instance, Burrows *et al*. (Burrows *et al*., 1989) reported that serum IgE could be predictive of rhinitis and asthma, on the contrary others (Hogan *et al*., 1997; Hogan *et al*., 1998) have shown that experimental asthma phenotype could be independent of IL-4 and allergen specific IgE. The correlation of allergen-specific IgE with the severity of allergic symptoms has also been shown to be weak (Niederberger *et al*., 2001).

Although our study showed that generally rhinitis subjects had higher allergen-specific IgE levels compared to non-rhinitis (Figure 3), allergen-specific IgE in serum is not the only indicator for allergic diseases as suggested by other investigators (Smith, 1992; Burrows *et al*., 1995; Droste *et al*., 1996). Therefore diagnosis by trained clinicians is still highly desirable (Smith, 1992). However, patients' sensitization profile can serve as helpful information in the diagnosis of rhinitis. This is because the odds of a rhinitis subject being sensitized to any of the three allergens used is generally greater than in non-rhinitis (Table 5). The association of mite sensitization with rhinitis was quite expected because various studies had indicated an association of rhinitis with

both pollen and house dust sensitization (Gergen & Turkeltaub, 1992; Boulet *et al*., 1997; Plaschke *et al*., 2000). Nonetheless, it should also be noted that antigen-specific IgE is not the most important causing factor in allergic rhinitis or allergic asthma. T_H2 cell and cytokine signalling pathways play a major role in the pathogenesis of allergic diseases (Corry, 2002). The production of antigen-specific IgE could be an important indication in the early stage of disease but not in the later stage.

3.5.2 Sensitization profile of Malaysian patients with asthma

The sensitization profile of Malaysia asthmatic adults, as determined by ELISA, was quite different to that of children asthmatics, especially with respect to Blo t 5 sensitization (Figure 5). This observation was probably attributed by the age factor (Boulet *et al*., 1997). Influence of age on IgE responsiveness to domestic mite allergens had been reported (de Groot *et al*., 1990; Shibasaki *et al*., 1994; Boulet *et al*., 1997). Though the frequency has decreased, it is still quite high in adults. Although this observation was in contrary to the report that sensitization actually increased with age (Gerritsen *et al*., 1990), it was in good accordance with the observation by Burrows *et al*. where they showed that serum IgE levels decline significantly with age among patients with asthma ($p<0.0001$) (Burrows *et al*., 1989). Furthermore, Boulet *et al*. also showed that allergen sensitization among older adults (>46 years old) was much lower than that of age group 6-15 years (Boulet *et al*., 1997). An earlier report by de Groot *et al*. also showed that children below age of four had a higher sensitization frequency towards domestic mites allergens and animal danders (de Groot *et al*., 1990). These could probably help to explain our observation because the age of adult patients with asthma in our study was generally skewed towards middle age rather than in the early twenties. Moreover, the patients in this study were all suffering clinician-

diagnosed asthma, and were not complicated with COPD which could have complicated the interpretation of the data (Dow, 1998).

The sensitization profiles of Malaysian children with asthma observed in this study revealed that sensitization to Blo t 5 allergen was as high as 90%, and sensitization to Der p 1 was 57%. The data are in good accordance with results previously reported by Leung *et al*. (Leung *et al*., 1997). It was shown that the frequency of positive skin prick test reactivity of older children (age range 12-18 years) in the general population from Kota Kinabalu, Sabah, Malaysia to *Der p* extract was about 60%. An interesting observation from another study was that allergic subjects having both asthma and rhinitis had significantly higher reactivity to Der p 5 compared to groups suffering from either of the diseases (Lin *et al*., 1994). Whether the asthmatic children in our study were also suffering from rhinitis were not noted. Therefore, we could only speculate that it might be the special properties of group 5 allergens that caused these observations (although previous study has demonstrated low crossreactivity between Blo t 5 and Der p 5) (Kuo *et al*., 2003).

Another worth noting observation in this study was that the sensitizations to Der p 2 were below 50% in both Singapore and Malaysian subjects. Our study coincided well with similar study conducted by other investigators in Thailand where they observed less than 50% sensitization to Der p 1, Der p 2 and Blo t 5 (Trakultivakorn & Nuglor, 2002). Nonetheless, this was rather surprising as the previous epidemiological study indicated that the extremely high number of both *Der p* and *Blo t* mites were found in Singapore homes (Chew *et al*., 1999 (b)) and the levels of *Blo t* and *Der p* allergens found in these homes were very high (Zhang *et al*., 1997). This result also showed discrepancy with other published studies in which it was reported that about 80% of the mite allergic asthmatic patients were sensitized to either

Der p 2 (Tsai *et al*., 2000) or Blo t 5 (Manolio *et al*., 2003). Therefore, further studies are required to fully address this discrepancy if the observed phenomenon was unique to this region of the world.

Although in general ELISA and SPT results did coincide, discrepancies did occur too (Table 6). This was rather expected because of the differences in nature of both tests (Smith, 1992). Besides that, the cut off for allergen-specific IgE in *in vitro* assays is still debatable (Bernstein & Storms, 1995). In relation to this issue, borderline cases could be misclassified as either positive or negative depending on the cut off value used (Homburger & Jacob, 1982).

3.5.3 Implications of mite sensitization in allergic rhinitis and allergic asthma

The association between mite sensitization and rhinitis was quite clear (Table 4, Table 5). Although similar analysis was not performed on allergic asthma subjects due to the lack of enough normal subjects for comparison, it would be expected that the association does occur as well for allergic asthma. The association of mite sensitization to allergic diseases has several implications. The first one being that it could be possible to reduce the symptoms of allergic diseases in patients by implementing allergen avoidance measures (Eggleston & Bush, 2001). The second would be allergen-avoidance among non-sensitized subjects could potentially reduce their chance of developing allergic diseases later in life. Nevertheless, the effectiveness of allergen avoidance on allergic disease control is still quite debatable (Boner *et al*., 2002).

3.5.4 Component-resolved diagnosis of mite sensitization

Component-resolved diagnosis (Valenta et al., 1999 (a)) of mite sensitization allows the identification of the major sensitizing mite allergens of a patient. Conventional crude mite extract could only indicate that whether one is sensitized to mite but could not pin point the exact allergen in the mite extract that causes the sensitization (Valenta & Kraft, 1995). Moreover, it is difficult to standardize the quality of the crude mite extract (Yunginger & Adolphson, 1992).

The individual allergens (Blo t 5, Der p 1, Der p 2) used in this study provided valuable insight into the mite sensitization profile of allergic rhinitis and allergic asthma subjects. It shows that Blo t 5 is a very important sensitizing agent in both disease groups (Figure 4; Figure 5). Between Der p 1 and Der p 2, it seems that Der p 2 is more important (Figure 4; Figure 5). It is possible that both Blo t 5 and Der p 2 are important early sensitizing allergens. This information can never be obtained using crude mite extract alone.

With the identification of the sensitizing allergens, it is then possible to perform component-resolved immunotherapy on these patients (Valenta *et al*., 1999 (a)).

3.6 *Conclusion and future direction*

In summary, our study confirms the results of a previous study (Kuo *et al*., 1999) that Der p 1, Der p 2 and Blo t 5 are important sensitizing allergens among the Singapore and Malaysian subjects. Besides, we also demonstrated the association between mite sensitization and rhinitis. For the Malaysian patients with asthma, sensitization to these three mite allergens was much more prevalent in children as compared to the adult counterparts. The three allergens are important reagents for component-resolved diagnosis of mite sensitization.

Future studies using recombinant and purified allergens should include allergic disease patients from other parts of South East Asia like Thailand, Indonesia, Vietnam, Myanmar, so as to give a better picture of the sensitization profile of allergic subjects in this region. Apart from that, the importance of Der p 1, Der p 2 and Blo t 5 in this part of the world could also be further established.

In this study, we did not look at the frequency of asthmatic symptoms among the allergic rhinitis subjects and vise versa. It would be interesting to do so in future studies.

Chapter 4

4 Cloning of a unique allergen from *Blomia tropicalis* and monoclonal antibody production

4.1 *Objectives and significance of the study*

This study contributed to the effort of identifying a complete spectrum of allergens from *Blo t* by identifying a unique allergen, Blo t 19, from *Blo t* via cDNA library screening.

Monoclonal antibody was proven to be useful in purifying native allergens from mite crude extracts (Tsai *et al*., 1998 (b); Ramos *et al*., 2003; Yang *et al*., 2003). Therefore, it was hoped that by generating monoclonal antibody against Blo t 19, its native form could be isolated.

A monoclonal antibody was successfully raised against Blo t 19. This monoclonal antibody was useful in western blot and ELISA.

Aside from the main objectives, this study also further proved the observation that different mouse strain and the nature of individual mite allergen could influence the outcome of immunization.

4.2 *Materials and methods*

4.2.1 Materials

4.2.1.1 **Primers**

Primer name	5' Primer sequence 3'
BspE1-Bt19	AAT CCGGA GCT CTC GAC TTT ACC AGC TGT
Bt19-Not I	ATA AGA ATG CGG CCG CTT AAC CCC TGG AGG GCA GAT T
M13 Forward (-20)	GTA AAA CGA CGG CCA G
M13 Reverse	CAG GAA ACA GCT ATG AC
pC1NeoF	TTAATACGACTCACTATAGG
pC1NeoR	CATTAACCCTCACTAAAGGG

Table 7: Primers used in this chapter. Underlined sequences are the restriction enzyme sequences introduced. All primers were purchased from Proligo Singapore Pty Ltd.

4.2.1.2 **Kits**

The following commercial kits were used: Wizard® Lambda Preps DNA Purification System kit (Promega, Madison, USA), QIAGEN Plasmid Giga, QIAquick® Gel Extraction Kit (QIAGEN, Hilden, Germany), DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Diagnostics, Manheim, Germany), PUREGENE™ DNA Purification Kit (Gentra Systems, Minneapolis, MN, USA), *Pichia* expression kit, TOPO-TA Cloning Kit (Invitrogen, Carlbad, CA, USA), Quick Ligation Kit (New England Biolabs Inc., Beverly MA, USA), ClonaCell™-HY Hybridoma Cloning Kit (StemCell Technologies Inc., Vancouver, Canada).

4.2.1.3 **Centrifugations**

All centrifugations were carried out in a table top centrifuge (Eppendorf Centrifuge 5415, Eppendorf AG, Hamburg Germany) unless otherwise specified.

4.2.1.4 **Serum samples**

Sera from 4 adults who showed positive skin prick test reaction to *Blomia tropicalis* extract were pooled and used to screen the *Blomia tropicalis* cDNA λgt11

library (4.2.2). 20 sera from candidates with positive skin prick test to *Blo t* extract were also chosen for IgE reactivity study (4.2.2.2). Another 22 sera (12 rhinitis patients, 10 healthy subjects) were also used to test the allergenicity of Blo t 19.

4.2.2 Identification of Blo t 19

4.2.2.1 Screening of λgt11 expression library of *Blomia tropicalis*

Library screening was performed by plaque IgE immunoassay. This cDNA library was generated from the local *Blomia tropicalis* mites. The pooled sera was adsorbed with lysate prepared from *E. coli* (Y1090) and recombinant *E. coli* containing GST-Blo t 5 overnight at 4°C on an orbital rotator before use. Phages were plated at 5-10,000 plaque-forming units (pfu) per 145 mm NZY agar plate (Appendix A). Plaques were transferred to nitrocellulose filters (Amersham Biosciences, Buckinghamshire, England) that were saturated with isopropyl-β-D-thiogalactopyranoside(IPTG). This was done by overlaying the IPTG treated filters on the growing plaques, and then incubating at 30°C for an hour in an incubator. The filters were then removed from the agar plate and after blocking the filters with 5% solution of skim milk powder for 1 hour, they were then washed three times with PBS-Tween 20 (0.05%, v/v) (washing solution), followed by overnight incubation with sera (1:1 dilution in washing solution containing 1% skim milk and 0.02% sodium azide) at 4°C. These filters were again washed three times. They were then incubated with the monoclonal anti-human IgE alkaline phosphatase conjugated (1:1,000 dilution) (Pharmingen, San Diego, CA, USA) for an hour at room temperature followed by three washes in buffer. After the last wash the filter was blotted on paper towels and then incubated in developing solution containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma) and nitro blue tetrazolium (NBT, Sigma), for half to an hour. An intense bluish purple colour reaction signal indicates a positive reaction. The clone that reacts positively in the first round

was subjected to several additional rounds of plating and screening to obtain a pure single plaque.

4.2.2.2 Human IgE reactivity of Blo t 19 clone of *Blomia tropicalis*

Individual serum from each candidate was treated as described in section 4.2.2 for pooled sera. A positive clone designated as Blo t 19 gt11 clone was plated at approximately 1,000 pfu per 145 mm NZY agar plate (Appendix A) and treated as previously described (4.2.2). Sera from 20 *Blo t* extract sensitized subjects were tested.

4.2.2.3 Isolation of DNA from the positive λgt11 cDNA clone, Blo t 19

Phages from immuno-positive plaques were amplified by the liquid lysate method. DNA was isolated using the Wizard® Lambda Preps DNA Purification System kit (Promega, Madison, USA) according to the manufacturer's instructions.

4.2.2.4 Polymerase chain reaction (PCR) amplification of phage clones

Approximately 40-60 ng of DNA was added in a 25 µl reaction mixture containing 0.4 pmol of λgt11 forward and reverse primers, 0.2 mM of dNTPs, 1 U of Native pfu DNA polymerase, 2.5µl of 10X Native Pfu DNA polymerase buffer (Stratagene, La Jolla, CA, USA). Forty cycles of amplification was performed using a GeneAmp PCR System 2400, Perkin Elmer. Each cycle consists of 95°C for 1 minute 30 seconds, 65°C for 1 minute 30 seconds and 72°C for 2 minutes. The PCR DNA was further purified using QIAquick® Gel Extraction Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol (4.2.2.5).

4.2.2.5 QIAquick® Gel Extraction Kit (QIAGEN, Hilden, Germany)

DNA was further purified using QIAquick® Gel Extraction Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Briefly, the DNA fragment was excised from the agarose gel and dissolved in 1 gel volume of Buffer QC at 50ºC for 10 minutes. After dissolving the gel, 1 gel volume of isopropanol was added and transferred to a QIAquick spin column in a 2.0 ml collection tube. The column was centrifuged at top speed for 1 minute. Flow-through was discarded and 0.75 ml of Buffer PE added to the column. The column was then centrifuged again with the same parameters as before. The flow-through was again discarded and the column was centrifuged for further 1 additional minute to remove the traces of PE buffer.

After that, the QIAquick column was transferred to a clean microcentrifuge tube. DNA was eluted by adding 30-50 µl of Milli-Q water to the centre of the QIAquick column and the column was then centrifuged at top speed.

4.2.2.6 Sequence Analysis

The deduced nucleotide sequence of Blo t 19 was submitted to the databases of the National Center for Biotechnology Information (NCBI), using the BLAST network server for sequence and amino acid homology search. Blo t 19 was subsequently cloned into *E. coli* (pGEX4T1-Blo t 19) and in yeast for recombinat protein expression. This was done by another colleague.

4.2.2.7 **Southern blot analysis**

4.2.2.7.1 ***Probe generation***

Blo t 19 gene was released from pGEX4T-1-Blo t 19 plasmid using BamHI and EcoRI restriction enzymes. The gene was gel purified from 1% agarose gel as described previously (4.2.2.5).

4.2.2.7.2 ***Probe labeling***

The Blo t 19 DNA probe was labeled using DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Diagnostics, Manheim, Germany) according to the manufacturer's recommendations. In brief, 1 µg of purified Blo t 19 gene, diluted to a total volume of 16 µl in ddH_2O, was heat denatured in boiling water bath for 10 minutes and immediately chilled on ice. To each reaction, 4 µl of the DIG-High Prime (Roche Diagnostics, Mannheim, Germany) was added, gently mixed, and incubated at 37ºC for 14 to 16 hours. Two microliters of 0.2 M Disodium ethylenediaminetetraacetate (EDTA) (BioRad, Hercules, CA, USA), pH 8.0, were added to each reaction tube to terminate the labeling reaction. The reaction was heated for 10 minutes at 65°C after the addition of EDTA.

4.2.2.7.3 ***Isolation of mite genomic DNA***

Genomic DNA purification was carried out according to the manufacturer's recommendation (PUREGENE™ DNA Purification Kit, Gentra Systems, Minneapolis, MN, USA). Briefly, 50-100 mg of frozen mites (*Blo t* or *Der f*) were homogenized thoroughly using 30-50 strokes of tube pestle in 3 ml of Cell Lysis Solution. The lysate was then incubated at 65ºC for 60 minutes and mixed by inverting 25 times or until tissue particulates have dissolved. 1.5 µl of RNase A Solution (4 mg / ml) was added to the lysate and mixed by inverting for 25 times and incubation at 37ºC for 60 minutes.

After that, the samples were cool to room temperature before 100 µl of Protein Precipitation Solution was added. The mixture was vortexed vigorously at high speed for 20 seconds followed by centrifugation at top speed (Jouan Centrifuge BR4i) for 3 minutes and the supernatant was transferred to a clean 1.5 ml microfuge tube containing 300 µl of 100% isopropanol. The mixture was mixed by inverting gently for 50 times followed by centrifugation at top speed for 5 minutes. Supernatant was removed and the DNA pellet washed by adding 300 µl of 70% ethanol. Then, centrifugation was again carried out at top speed for 1 minute at 4ºC. The supernatant was aspirated dry and the pellet air-dried for 10-15 minutes before being dissolved in 50 µl of DNA Hydration Solution for 1 hour at 65ºC. The DNA was stored at -20ºC until use.

4.2.2.7.4 ***Hybridization and detection***

Hybridization and detection was carried out according to the manufacturer's recommendation (DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Diagnostics, Manheim, Germany)). Mock hybridization (hybridizing empty membranes with different concentrations of probe) was carried out to determine the optimum probe concentration for hybridization.

4.2.2.8 **Allergenicity of GST-Blo t 19**

ELISA was performed as described in section 3.3.3 with the following modifications: sera (1:5 dilution) was tested against GST-Blo t 19 and Glutathione S-transferase (GST). Sera from 12 Blo t extract sensitized rhinitis subjects were tested. The cut off value was derived from mean plus two standard deviations of readings from 10 healthy subjects.

4.2.3 Cloning

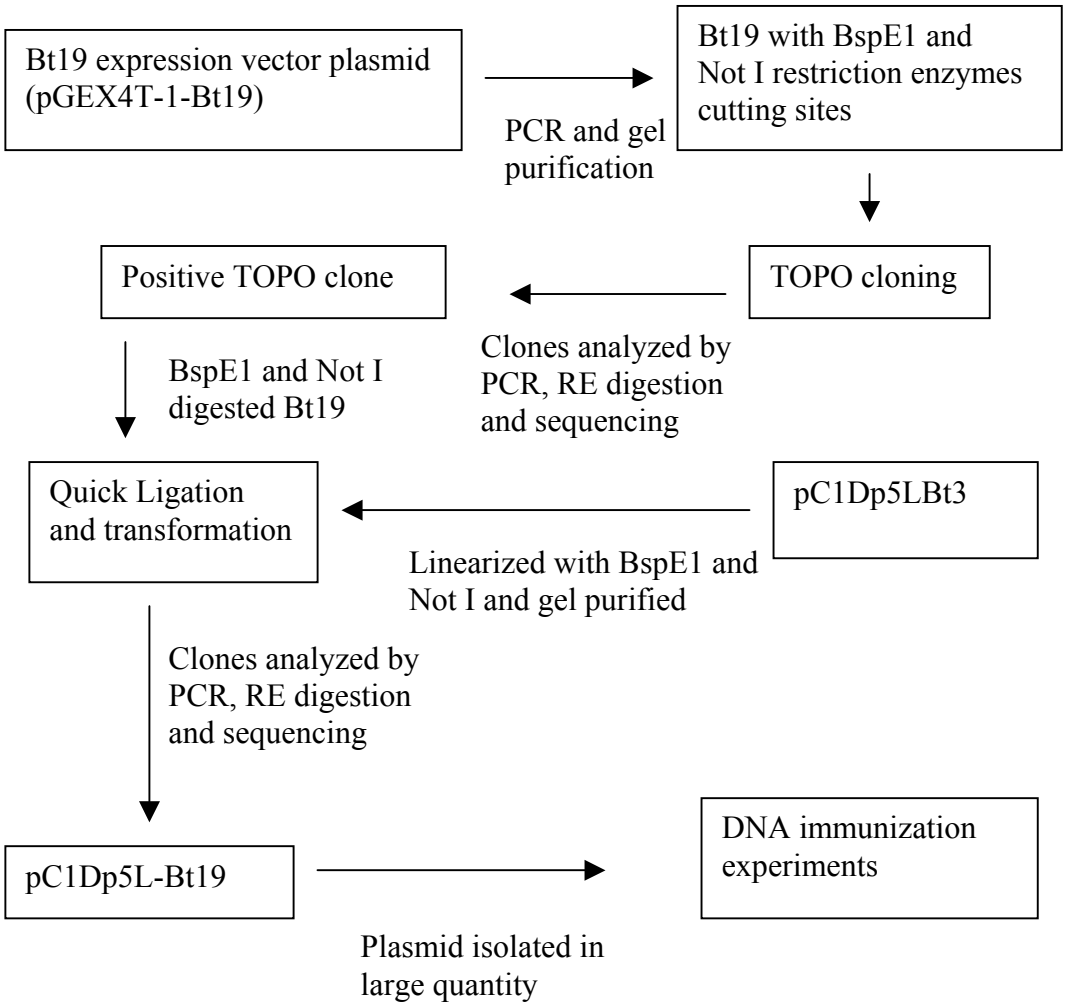

Figure 6: Flow chart of the cloning strategy employed in this chapter. Note: PCR: polymerase chain reactions; RE: restriction enzyme; Bt19: Blo t 19. RE digestion was performed using BspE1 and Not I restriction enzymes. Primers used to amplify Blo t 19 gene from pGEX4T-1-Bt19 were BspE1-Bt19 and Bt19-Not I (Table 7).

The cloning processes of Blo t 19 gene into pC1Dp5L vector were shown in Figure 6.

4.2.3.1 **Cloning of Blo t 19 from pGEX4T-1 to pCR2.1 (TOPO vector)**

BspE1 and Not I restriction enzyme cutting sites were introduced at the 5' and 3' end of the Blo t 19 gene amplified from pGEX4T-1-Bt19 plasmid using BspE1-Bt19 and Bt19-Not I as primers (Table 7) in PCR (Figure 6). PCR products were analyzed on 1% agarose gel electrophoresis and purified using QIAquick® Gel Extraction Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol (4.2.2.5).

Cloning was performed according to the manufacturer's recommendations (Invitrogen, Carlbad, CA, USA). Briefly, a TOPO cloning reaction (6 µl) which consisted of 2 µl of fresh PCR product, 1 µl salt solution, 2 µl of sterile Milli-Q water and 1 µl of TOPO vector (Appendix B), was set up. The mixture was gently mixed and incubated for 30 minutes at room temperature (~25°C). After that, the reaction mixture was placed on ice and transformed into competent cells using Top 10 One Shot Chemical Transformation protocol (4.2.3.2).

4.2.3.2 **Top 10 One Shot Chemical Transformation**

The protocol was performed according to the manufacturer's recommendation (Invitrogen, Carlbad, CA, USA). Briefly, 2 µl of the TOPO reaction was added into a vial of One Shot Chemically Competent *Escherichia coli* (Invitrogen, Carlbad, CA, USA) and mixed gently. The mixture was incubated on ice for 30 minutes, followed by 30 seconds of heat shock in 42°C water bath, and placed on ice again. Then, 250 µl of room temperature SOC medium was added. The tube was capped tightly and incubated in a 37°C shaker incubator for 1 hour. 50-200 µl of each transformation mixture was

spread onto selective plate and incubated overnight at 37°C. The next day, white colonies (positive) were picked for analysis.

4.2.3.3 **Cloning of Blo t 19 from TOPO vector into pC1Dp5L vector**

pC1Dp5L vector was chosen based on similar experiment as previously decribed by Yang and colleagues (Yang *et al*., 2003). It was shown that with pC1Dp5L vector carrying the Blo t 3 gene was able to induce immune response in mice which subsequently enabled Blo t 3-specific monoclonal antibody to be produced (Yang *et al*., 2003).

Blo t 19 gene was released from TOPO vector using BspE1 (New England Biolabs Inc., Beverly MA, USA) and Not I (Promega, Madison, USA) restriction enzymes. Then, Blo t 19 gene was ligated into BspE1 and Not I digested pC1Dp5L vector (Appendix B) using Quick Ligation Kit (New England Biolabs Inc., Beverly MA, USA) according to the manufacturer's instruction.

Briefly, 50 ng of vector was combined with a 3-fold molar excess of insert in a total of 10 µl reaction mixture (top up with Milli-Q water). 10 µl of 2X Quick Ligation Buffer (New England Biolabs Inc., Beverly MA, USA) was added and mixed. Then, 1 µl of Quick T4 DNA Ligase (New England Biolabs Inc., Beverly MA, USA) was added and mixed thoroughly. The reaction was then centrifuged briefly and incubated at room temperature (25°C) for 5 minutes, followed by chilling on ice and transformed according to manufacturer's recommendation.

50 µl of competent cells was added to 2 µl of chilled ligation mixture in a 1.5 ml microcentrifuge tube, mixed by pipeting up and down, and incubated for 30 minutes. After that, the mixture was subjected to heat shock for 2 minutes at 37°C and chilled on ice for 5 minutes. Then 950 µl of room temperature LB broth was added and

incubated for 37°C for one hour. Next, 100 μl of the mixture was spread onto LB agar plate containing ampicillin (CalBioChem®, EMD Biosciences, Inc., San Diego, CA, USA) and incubated overnight at 37°C.

4.2.3.4 **Polymerase chain reactions (PCR)**

Polymerase chain reactions (PCR) were carried out in thermal controller (PTC-100™ programmable thermal controller, MJ Research Inc.). A typical PCR reaction was as follow:

Components	**Volume (μl)**
Milli-Q water	17.25
10X buffer	2.5
25 mM $MgCl_2$	1.5
Forward primer (10 pmol / μl)	1.0
Reverse primer (10pmol / μl)	1.0
10mM dNTPs	0.5
Mix well, followed by short centrifuge	
Taq polymerase	0.25
DNA template	1.0
Total	25.0

Table 8: Typical PCR reaction used in the study. Forward primer was BspE1-Bt19 (Table 7) and reverse primer was Bt19-Not I (Table 7).

Each cycle of PCR consisted of 94°C for 15 seconds followed by 55°C for 30 seconds followed by 72°C for 1 minute. Thirty cycles were carried out with a final 72ºC for 7 minutes at the end of the thirty cycles.

4.2.3.5 **DNA sequencing**

Typical PCR reaction set up was as follow:

Components	Volume (μl)
Milli-Q water	10.0
Big Dye ® sequencing mix (Applied Biosystem, Foster City, CA, USA)	8.0
Forward / Reverse primer (10 pmol / μl)	1.0
DNA template	1.0
Total	20.0

Table 9: Typical PCR reaction used in this study for sequencing (according to manufacturer's recommendation). Forward primer was M13 forward (-20) (Table 7) and reverse primer was M13 reverse (Table 7) for sequencing TOPO clones. For sequencing pC1Dp5L-Bt19 clones, pC1NeoF (Table 7) and pC1NeoR (Table 7) was used as forward and reverse primers.

Each cycle of PCR consisted of: 96°C for 30 seconds, 50°C for 15 seconds and followed by 60°C for 4 minutes. The reaction was subjected to 26 times of this cycle. Reactions were carried out in thermal controller (PTC-100™ programmable thermal controller, MJ Research Inc.).

4.2.3.6 **Ethanol precipitation**

Two microliters of 3 M sodium acetate, pH 4.6, and 50 μl of cold absolute ethanol were added to the PCR product. The mixture was vortexed and placed on in -80°C freezer for 15 minutes prior to centrifugation at 14,000 rpm using a tabletop centrifuge, 5415C (Eppendorf, Hamburg, Germany) for 20 minutes at 4°C. The ethanol was aspirated with a micropipettor and the pellet, rinsed with 250 μl of 70% pre-cooled ethanol and centrifuged as mentioned. Then, the ethanol was aspirated and the pellet was air-dried.

4.2.3.7 **Alignment**

All alignment of sequences was performed using Clustal W Multiple Sequence Alignment Program version 1.8 (http://clustalw.genome.ad.jp/).

4.2.3.8 **Large scale preparation of pC1Dp5L-Bt19 DNA for injection**

pC1Dp5L-Bt 19 plasmid was prepared in large scale using QIAGEN Plasmid Giga (QIAGEN). Briefly, single colony picked from freshly streaked selective plate was used to inoculate a starter culture of 10 ml LB broth medium (Appendix A) containing the ampicillin (CalBioChem®, EMD Biosciences, Inc., San Diego, CA, USA). The culture was incubated for around 8 hours at 37°C with vigorous shaking (~300 rpm), after which the culture was diluted 500 to 1000 times into 2.5 litres of selective LB medium and grown at 37°C for 12-16 hours with vigorous shaking (~300rpm). The bacterial cells were harvested by centrifugation at 6000 x g for 15 minutes at 4°C. Then, the bacterial pellets were resuspended in 125 ml of Buffer P1, followed by 125 ml of Buffer P2. The mixture was mixed gently by inverting 4-6 times, and incubated at room temperature for 5 minutes. After that, 125 ml of chilled Buffer P3 was added and the mixture mixed immediately but gently by 4-6 times of inversion and incubated on ice for 30 minutes. After incubation, the mixture was centrifuged at ≥20,000 x g for 30 min at 4 °C. Supernatant containing plasmid DNA was removed promptly and re-centrifuged at ≥ 20,000 x g for 15 minutes at 4°C. Supernatant containing plasmid DNA was removed promptly and loaded to pre-equilibrated QIAGEN-tip 10000 column (equilibrated by passing 75 ml of buffer QBT through the column via gravity flow). The QIAGEN-tip was then washed with a total of 600 ml buffer QC. DNA was eluted with 75 ml Buffer QF.

DNA was precipitated by adding 52.5 ml (0.7 vol) room-temperature isopropanol (Merck) to the eluted DNA. The mixture was then mixed and centrifuged immediately at ≥15000 x g for 30 minutes at 4°C. The supernatant was decanted carefully. DNA pellet was washed with 10 ml of room temperature 70% ethanol, and centrifuge at ≥15000 x g for 10 minutes. Again the supernatant was decanted without

disturbing the pellet. The pellet was air-dried overnight, and redissolved in a suitable volume of sterile 1X PBS.

4.2.4 Monoclonal antibody generation

4.2.4.1 Mice

Six to eight weeks old female Balb/c, Balb/cJ, AKR, CBA, C57/B6 mice kept under conventional conditions were used for the various experiments in this chapter. Five mice per group were typically used for monoclonal antibody production experiments and three mice per group were used in mouse strain difference experiment. Animal experiments were performed according to Institutional Guidelines for Animal Care and Handling, National University of Singapore.

4.2.4.2 Immunization protocols

4.2.4.2.1 *DNA Delivery*

4.2.4.2.1.1 Intramuscular and electroporation

The experiment was carried out according to the protocol described by Widera and co-workers. (Widera *et al*., 2000). In summary, mice were anaesthetized using CRC cocktail (Appendix A), 1 µl / g of body weight delivered with 1 ml Tuberculin Latex Free Syringe and BD 27G1/2 PrecisionGlide Needle, (Becton Dickinson, Franklin Lakes, NJ, USA). Each mouse received 50 µg (in 50 µl) of DNA delivered intramuscularly in the posterior thigh. The skin on the tibialis anterior muscle was shaved before injection. After the intramuscular injection, two-needle array electrodes (5mm) were inserted (to a depth of around 2 mm) at both ends of the injected site immediately for electroporation. The electroporation parameters were: Voltage: 82V, pulse length: 20 ms, Pulse: 4, Interval: 200 ms on a BTX ECM830 square wave electroporator (BTX, Genetronix Inc., CA, USA). Mice were immunized according to

the immunization schedule (Figure 7). Blood was extracted from orbital sinus to monitor the titer of antibody production. Blood sera were stored at -80°C until used.

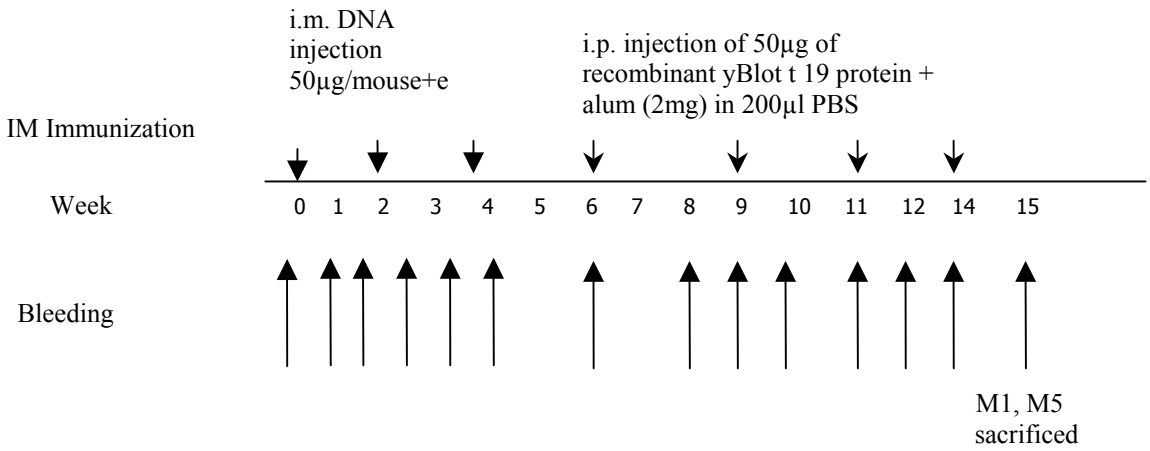

Figure 7: Immunization schedule for DNA immunization coupled with protein boost. Note: i.m.: intramuscular; i.p.: intraperitoneal; e: electroporation.

4.2.4.2.1.2 Intrasplenic injection of DNA

Intrasplenic injection was performed according to protocols described previously (Spitz *et al*., 1984; Velikovsky *et al*., 2000). Briefly, Balb/c mice were anaesthetized as described previously (4.2.4.2.1.1). The mice were placed on its right side. The fur from the left side was shaved and the abdomen swabbed with 70% ethanol. A skin incision 1-1.5 cm long was made on the skin, followed by the muscular wall and abdominal wall until the spleen was exposed. The spleen was exteriorized by gently lifting its lower pole, and moved a little further with the aid of forceps. The needle was inserted deeply into the spleen and the DNA injected as the needle was pulled out, in order to spread the DNA (50 µg in 50 µl) evenly through most of the spleen (Figure 8-9).

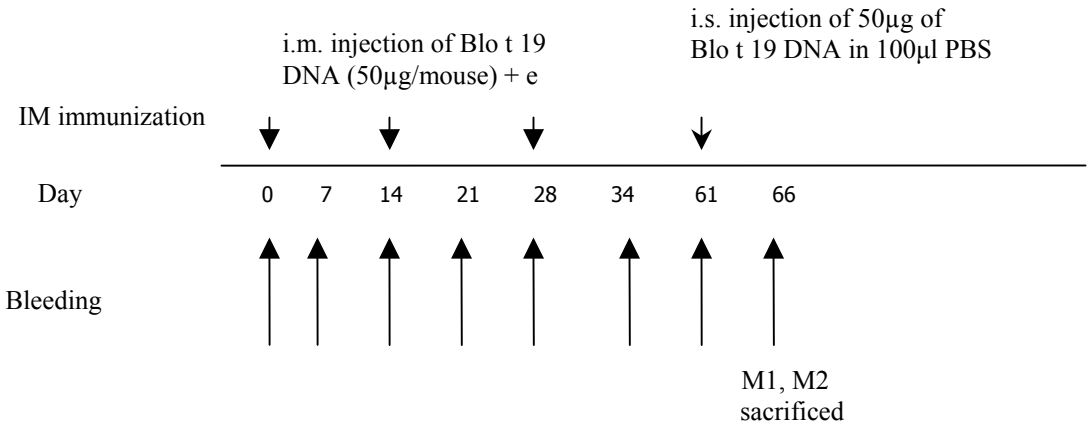

Figure 8: Immunization schedule of DNA immunization coupled with intrasplenic boost. Note: i.m. : intramuscular; i.s.: intrasplenic; e: electroporation.

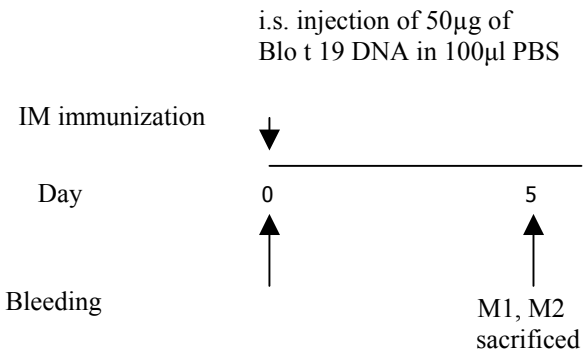

Figure 9: Immunization schedule with intrasplenic injection alone.

4.2.4.2.2 ***Protein Delivery***

4.2.4.2.2.1 Intraperitoneal (i.p.) with alum

200 µl of yeast-expressed Blo t 19 (250 µg / ml) containing 2 mg of alum (Whitehall Lab Pty Ltd., Punchbowl, Australia) was injected intraperitoneally into each mouse. This was performed as a booster shot to mice previously immunized with plasmid DNA expressing Blo t 19 (Figure 7).

4.2.4.2.2.2 Subcutaneous (s.c.) with Complete/Incomplete Freund's Adjuvant

A mixture of 1:1 of protein solution to complete Freund's adjuvant (CFA) (Gibco^{TM}, Invitrogen, Carlbad, CA, USA) was set up. Each mouse was to receive 20

μg of protein in CFA. The mixture was homogenized on ice using Microson™ ultrasonic cell disrupter (Misonix Inc., Farmingdale, NY, USA). Sonication was performed for 15 s, with 15 s intervals, until the mixture was homogenized. The same procedure was applied to protein + incomplete Freund's adjuvant (IFA) (Gibco™, Invitrogen, Carlbad, CA, USA).

The homogenized mixture of protein + CFA/IFA was transferred to a 1 ml Tuberculin syringe. Mice were anesthetized and each mouse received 100 μl of the mixture subcutaneously.

Time	Procedure performed
Day 0	Blood collection, s.c. injection of 20 μg yBlo t 19 + CFA/mouse.
Day 10	Blood collection, s.c. injection of 20 μg yBlo t 19 + IFA/mouse.
Day 20	Blood collection, s.c. injection of 20 μg yBlo t 19 + IFA/mouse.
Day 30	Blood collection.

Table 10: Immunization schedule of protein immunization. Each mouse was immunized with 20μg of yeast expressed Blo t 19 (yBlo t 19) coupled to either CFA/IFA subcutaneously.

4.2.4.3 Fusion using ClonaCell™-HY

Fusion was carried out according to the manufacturer's instruction (StemCell Tech Inc., Vancouver, BC, Canada, Vancouver, BC, Canada). Briefly, 2 x 10^7 of log phase growing myeloma cells (P3X63Ag8.653) (American Type Cell Culture (ATCC), Manassas, VA, USA) were resuspended in 30 ml Medium A (MA) (StemCell Tech Inc., Vancouver, BC, Canada) (prewarmed to 37°C). 1 x 10^8 viable spleen cells were added to the P3X63Ag8.653 myeloma cells (ATCC, Manassas,VA, USA) and centrifuged at 400 x g for 5 minutes. The supernatant was discarded, pellet broken up by tapping on the side of the tube, and washed twice with 40ml of prewarmed Medium B (MB) (StemCell Tech Inc., Vancouver, BC, Canada, Vancouver, BC, Canada). After

that, the supernatant was removed completely and the pellet was gently resuspended with 1 ml of PEG solution (prewarmed to 37°C) using 1ml pipet (Becton Dickinson and Company, Franklin Lakes, NJ, USA) over 1 minute, stir with pipet over 1-2 minutes. 4 ml of MB was added over 4 minutes. 10 ml of MB was then added. After that, the mixture was incubated for 5 minutes in 37°C water bath. After that the mixture was washed twice with 40 ml of MA. Then the pellet was resuspended with 10 ml Medium C (MC) (StemCell Tech Inc., Vancouver, BC, Canada, Vancouver, BC, Canada) and transferred to 250 ml tissue culture flask containing 40 ml MC for incubation overnight (16-24 hours), at 37°C.

The next day, the mixture of cells was centrifuged at 1200 rpm for 10 minutes. 0.5 ml of the supernatant was used to resuspend the pellet while the remaining supernatant was discarded. After that, 10ml of MA was added. Meanwhile, a bottle of prewarmed Medium D (MD) (StemCell Tech Inc., Vancouver, BC, Canada) was stirred well with 10 ml pipet and 10 ml of cells added to it. The mixture was incubated for 30 minutes in 37°C, 5% CO_2. After that, the mixture was plated out in ten 100 mm Petri dish. The plates were incubated in the CO_2 incubator for 14 days.

Two weeks later (10-14 days), colonies visible to the naked eye were picked and placed in 96-well plate (Nunc™, Nalge Nunc International Corp, Naperville, IL, USA) containing 200 µl of prewarmed Medium E (ME) (StemCell Tech Inc., Vancouver, BC, Canada) in each well.

4.2.4.4 **Screening of antibody secreting hybridoma clones**

4.2.4.4.1 ***ELISA for detection of poly/monoclonal antibody***

The ELISA protocol was similar to ELISA protocol described in 3.3.3 except that the allergens used were either GST-Blo t 19 (5 µg / ml), yBlo t 19 (5 µg / ml), GST and *Blo t* mite crude extract (200 µg / ml). The test medium was supernatant from

hybridoma clones (50 µl / well). Rat anti-mouse immunoglobulins (Sigma-Aldrich, Saint Louis, MO, USA) (1 : 5000) was used as secondary antibody. Optical density reading was read at 15 minutes and 30 minutes at a wavelength of 405 nm.

4.2.4.4.2 *ELISA for isotyping of antibody*

The ELISA protocol was similar to ELISA protocol mentioned in 3.3.3 except that the coating antigen were yBlo t 19 (5 µg / ml) and Blo t extract (200 µg / ml), the primary antibody was hybridoma supernatant and the secondary antibody used was either rat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM or IgE (Serotec, Oxford, UK) (working concentration: 250 ng / ml, rat anti-mouse IgA (Sigma-Aldrich, Saint Louis, MO, USA) (dilution factor: 1 : 10000) for the detection of the respective antibody isotype.

4.2.4.5 **Mantaining and expanding hybridoma clones**

Culture medium for hybridomas was changed when it turned from pink to yellow. 110 µl of the culture medium was collected from the wells for analysis using ELISA (4.2.4.4) and replaced with fresh ME.

Positive clones were expanded into 24-well plate. Briefly, the clones were resuspended in their original 96-well (Nunc™, Nalge Nunc International Corp, Naperville, IL, USA) and transferred to 24-well plate (Nunc™, Nalge Nunc International Corp, Naperville, IL, USA) containing 1 ml of ME and cultured over 1 to 2 days when additional 1 ml ME was added. Culture medium that turned yellow was collected for isotyping ELISA (4.2.4.4.2). Positive clones were further expanded into 6-well plates (Becton Dickinson and Company, Franklin Lakes, NJ, USA) and some were frozen (4.2.4.7). From 6-well, the clones were subsequently adapted to HT medium (Appendix A) and further expanded in T-25 cell culture flasks (Corning Inc.,

Corning, NY, USA) and subsequently to T-75 flasks (Corning Inc., Corning, NY, USA) for ascites production (4.2.6.4).

4.2.4.6 Subcloning by limiting dilution to purify monoclonal antibody producing clone

The day before the cloning, 24-well plates were fed with fresh medium. The next day, the cells were resuspended and 1 ml of the suspension was transferred a sterile 15 ml tube (Becton Dickinson and Company, Franklin Lakes, NJ, USA). 50 µl of this suspension was taken for cell viability count (viability should be >80%). For each hybridoma cell line, the dilutions to give 4 cells / ml, 2 cells / ml and 1 cell / ml in cloning medium were calculated. Serial dilutions were made accordingly to yield 4, 2, and 1 cell/ml. Then, the dilutions were plated out in 96-well plates (200 µl / well). The plates were incubated at 37^{o}C, 5% CO_2 for 5-7 days. With the help of microscope, the number of colonies formed was noted to determine cloning and plating efficiency. Refeeding was performed by adding 50 µl of fresh medium to wells with noticeable cells.

4.2.4.7 Freezing of myeloma cell-line P3X63Ag8.653 and hybridoma clones

Cell culture was resuspended and cell number counted using trypan blue exclusion method. The cell suspension was centrifuged at 400 x g for 8 minutes. Supernatant was discarded and the cell pellet was resuspended in fetal calf serum (FCS) (HyClone, Logan, Utah, USA). Then equal volume of FCS (HyClone, Logan, Utah, USA) containing 20% DMSO (Sigma-Aldrich, Saint Louis, MO, USA) was added to yield the final cell concentration of 5 x 10^6 to 1 x 10^7 cell / ml. The cell mixture was then dispensed into cryovials (1 ml / vial) (Nunc™, Nalge Nunc International Corp,

Naperville, IL, USA) and freeze at -20°C for half an hour, followed by -86°C overnight and finally transferred to liquid nitrogen for long term storage.

4.2.4.8 **Thawing of myeloma cell-line P3X63Ag8.653**

One vial of cells from -86°C or liquid nitrogen was thawed in 37°C water bath for a few minutes. Then the cell suspension was removed and added dropwise into a 20 ml room temperature MA. The suspension was then centrifuged at 400 x g for 8 minutes. The supernatant was discarded and the cell pellet resuspended in 30 ml of Medium A and transferred to a T-75 flask for incubation overnight at 37°C, 5% CO_2 incubator.

The second day, the suspension was resuspended and the flask was left to stand vertically in the incubator for 30-60 minutes. After that, around 20 ml of medium was removed and replaced with fresh medium. The cell culture was then left to incubate for a 2-3 days. The cell culture was split accordingly to yield the required number of cells for fusion.

4.2.4.9 **Thawing of hybridoma clones**

One vial of cells was thawed in 37°C water bath for a few minutes. The vial was removed from the water bath when the ice has just melted and the cell suspension was diluted by the dropwise addition of the cell suspension to an equal volume HT medium (Appendix B). The suspension was left to stand for 5 minutes before an equal volume of HT medium (Appendix B) was added. The suspension was left for a further 5 minutes and finally centrifuged at 400 x g for 5 minutes. After the supernatant had been discarded, the cell pellet was resuspended in 5 ml of HT medium and cultured in a T-25 flask (Corning Inc., Corning, NY, USA).

4.2.5 Mouse strain difference study

Different strains of mice were selected: Balb/c, Balb/cJ, AKR, CBA, C57/B6. Three mice from each strain were used for the experiment. Each mouse was treated with i.p injection of 50 μg of yBlo t 19 and alum three times within 4 weeks (one dose biweekly). Blood samples were collected for analysis on day 0, day 10 and day 17.

Besides that, 2 groups of mice (5 Balb/c and 5 Balb/cJ) were used to study the responses to i.m. injection of plasmid DNA (pC1-Derp5L-Blo t 19) with electroporation. The mice were treated with 50 μg / mouse of plasmid plus electroporation biweekly until each mouse received 3 doses of plasmid injections. Blood samples were collected for analysis weekly.

4.2.6 Identification and Purification

4.2.6.1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were analyzed by SDS-PAGE modified from the method previously described (Laemmli, 1970). In brief, protein samples were mixed 1:1 with 2X SDS-PAGE sample buffer (Appendix A) and were boiled for 10 minutes. Samples were separated on a 15 % Tris-Glycine (or other gel percentage as stated) denaturing gel (Appendix A) using the Mini PROTEAN electrophoresis system (BioRad, Hercules, CA, USA). Gel was run at 120 Volts for 90 minutes. Broad Range Marker (BioRad, Hercules, CA, USA) was used as standard. All SDS-PAGE gels were stained as described in (4.2.6.3).

4.2.6.2 **Western Blot**

Gel was run according to instructions from gel/apparatus manufacturer. Typically, gels were run at 110V constant voltage. Electrophoresis was stopped when the dye front was about 1 cm from the bottom of the gel.

Transfer of protein from gel to nitrocellulose membrane was performed according to manufacturer's instructions. After transfer, the membrane was blocked in blocking solution (1X PBS, 0.05% Tween 20, 5% Skim milk on a rocker platform for 60 minutes at room temperature. After that, the membrane was incubated in primary antibody (hybridoma supernatant / ascites / purified monoclonal antibody / biotinylated monoclonal antibody) diluted in blocking solution (1 : 1000 / 1 : 4000) on a rocker plate overnight at 2-8 ^{o}C.

The membrane was then washed 6 times for a minimum of 5 minutes each in wash solution (1X PBS, 0.05% Tween 20). After that, the membrane was incubated in secondary antibody conjugate (anti mouse total immunoglobulin biotin conjugated (1 : 5000) / anti mouse isotype biotin conjugated (1 : 2000) diluted in fresh wash solution) for 60 minutes at RT on a rocker plate. The wash process was then repeated, followed by the addition of ExtrAvidin® (alkaline-phosphatase and peroxidase-conjugated) (Sigma-Aldrich, Saint Louis, MO, USA), 1 : 5000 dilution in wash solution and incubation for 1 hour at room temperature on rocker.

After an hour, the membrane was washed as previously described. Substrate (SuperSignal® West Pico Chemiluminescent Substrate, Pierce, Rockford, IL, USA) was then added, followed by incubation time of 5 minutes on rocker.

For development, the membrane was wrapped in plastic that fits in the cassette for film development, with excess substrate removed. BioMax Film (Eastman Kodak

Company, Rochester, NY, USA) was exposed to the membrane in dark room. Exposure time was reduced or increased accordingly to reduce unspecific signal.

For colorimetric reaction, the membrane was removed from the plastic and rinsed with Milli-Q water followed by 2X with wash solution for 5 minutes each. It was then incubated for 20-30 minutes with 20 ml (big Petri dish) 1x AP buffer containing 200 µl each of AP color reagent A and B (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was washed with Milli-Q water and dried for record when satisfactory signal intensity was observed.

4.2.6.3 **Gel staining**

All the steps were performed with constant shaking at room temperature (~25ºC) unless otherwise specified.

4.2.6.3.1 ***Coomasive blue staining***

SDS-PAGE gel was rinsed briefly in water. Then the gel was immersed in around 100 ml of Coomasive blue stain for ½ to 1 hour. After that, the gel was destained using around 100 ml of destaining solution for ½ to 1 hour. The destaining solution was then replaced and destained for a further of 1 to 2 hours, after which, the destaining solution was replaced with Milli-Q water. The gel was soaked in around 100 ml of Milli-Q water with two to three changes, 30 minutes between each change, before it was left overnight in Milli-Q water. The gel was framed and dried overnight the next day.

4.2.6.3.2 ***Silver staining***

SDS-PAGE gel was fixed in 200ml of fixing solution (50% (v/v) methanol, 5% (v/v) acetic acid in water) for 30 minutes. Then, the gel was washed with 200ml of 50% (v/v) methanol in water for 10 minutes, followed by washing in 200 ml of Milli-Q

water. After that, the gel was sensitized in 200ml of sensitizing solution (0.02% (w/v) sodium thiosulfate (Merck, Darmstadt, Germany) for 2 minutes. Then, it was washed with 200 ml of Milli-Q water twice, 1 minute each. After removing the water, the gel container was wrapped in aluminum foil to protect from light. 50 ml of chilled 0.1% silver nitrate ($AgNO_3$) was then added and incubation was carried out in 4ºC for 40 minutes with constant shaking. Thereafter, the gel was washed twice with 200 ml Milli-Q water, 1 minute each.

Development was performed by replacing the Milli-Q water with 100 ml of 2% sodium bicarbonate ($NaHCO_3$) (Sigma-Aldrich, Saint Louis, MO, USA) containing 0.04% (v/v) formalin (37% formaldehyde in water) (Sigma-Aldrich, Saint Louis, MO, USA) and constantly shaken for 15 s. Then, the developing solution was replaced with 100 ml of fresh developing solution and incubated until bands with the required contrast appeared (5-10 minutes). Then, the developing solution was discarded and replaced with 200 ml of stop solution (3.65 g / 250 ml $Na_2EDTA.2H_2O$) and incubated for 10 minutes. The gel was then washed thrice with 200 ml of Milli-Q water, 10 minutes each. After this, the gel could either be framed.

4.2.6.4 **Ascites production**

All animal handling were performed in compliance with local regulations. Mice (Balb/c) were primed by intraperitoneal (i.p.) injection of 0.5 ml of Pristane (Sigma-Aldrich, Saint Louis, MO, USA) 1-2 weeks before cell injection. Hybridoma cells grown in T-75 culture flasks (Corning Inc., Corning, NY, USA) were harvested in log phase and the viability by trypan blue exclusion. The cells were centrifuge at 1000 x g for 10 minutes at 20˚C. The pellet was washed twice in PBS (20 ml each time) and concentration adjusted to 5 x 10^6 / ml PBS.

5 x 10^6 cells in 1 ml PBS was injected i.p. into each mouse via the lower left quadrant from the midline. After around 10 days, when liquid tumor formation was noticeable (stomach of mice started bulging), ascitic fluid was extracted from the peritoneal cavity with an 18-G needle over a 15 ml tube. The ascites was left at room temperature for around 1 hour at 37ºC. Then, the fluid was kept overnight at 4ºC. The next day, the ascites was centrifuged at 3000 x g, 10 minutes, at 4˚C to remove cells and debris, and the clear supernatant was collected (discarding oil). The clear supernatant was aliquoted in 1 ml each in 1.5 ml microcentrifuge tube and stored at -80˚C. Mice were tapped 2-3 times (every 2-3 days) before being sacrificed.

4.2.6.5 **Biotinylation of monoclonal antibody**

Purified monoclonal antibodies (AF6 and I3D3) were diluted with coupling buffer to a concentration of 2 mg / ml (total volume 500 µl). An aliquot of 50 µl of biotinylation reagent (Biotinamidocaproate N-hydroxysuccinimide ester (Sigma-Aldrich, Saint Louis, MO, USA), 1 mg / ml in DMSO (Sigma-Aldrich, Saint Louis, MO, USA) was added to each antibody preparation. The biotin:antibody ratio used was approximately 10:1. The mixture was incubated overnight at 4°C with constant gentle shaking. To stop the reaction, 10 µl of 16.7 M ethanolamine (Sigma-Aldrich, Saint Louis, MO, USA) was added and the mixture was gently mixed at room temperature (22-25ºC) for 2 hours. The mixture was then dialyzed extensively in coupling buffer at 4°C to remove excess biotin. The biotin-conjugated antibodies were stored in aliquots in -20ºC until use.

4.2.6.6 **Monoclonal antibody specificity determination**

The specificity of the antibody was determined using direct ELISA and absorption studies (4.2.6.6.1-3).

4.2.6.6.1 *Direct ELISA*

ELISA plate was coated with either 500 ng of yBlo t 19, 20 µg Blo t extract or coating buffer alone. Primary antibody used was hybridoma supernatant from AF6 hybridoma. The supernatant was used without any dilution. The secondary detection antibody was rat anti-mouse IgG1 biotin conjugated (1 : 2000). Other than the above modification, the ELISA protocol was as previously described (3.3.3).

4.2.6.6.2 *Absorption study using ELISA*

The ELISA protocol was as previously described (3.3.3) except for the following modifications. Briefly, AF6-biotin (1 : 4000) was dispensed 50 µl / well into plates coated with 500 ng / well of GST-Blo t 19, yBlo t 19, GST or coating buffer alone (blocked and washed as previously described (3.3.3)) and incubated overnight at 4ºC. The next day the liquid from each well was transferred to another plate coated with the either GST-Blo t 19 or yBlo t 19 and incubated overnight. The plate was then washed and ExtrAvidin® alkaline phosphatase conjugate (Sigma-Aldrich, Saint Louis, MO, USA) (1 : 2000) was added into each well (50 µl / well) and processed accordingly as previously described (3.3.3).

4.2.6.6.3 *Absorption study using western blot*

1.3 µg of purified AF6 was incubated overnight with either 20 µg or 5 µg of GST-Blo t 19 or 1X PBS overnight at 4ºC on a rotater with constant rotation. These mixtures were later diluted 1000X to be used as primary detection antibody in western blot (4.2.6.2).

4.2.6.7 **Purification of monoclonal antibody from ascites**

4.2.6.7.1 ***Packing of Protein G column***

The Protein G column was packed according to the manufacturer's instruction. Briefly, frit was soaked in 20% ethanol. It was pressed to remove trapped air. The barrel of the column (Pierce, Rockford, IL, USA) was half filled with wash/binding buffer. The soaked frit was then pushed into the barrel until it rested firmly on the bottom. The cap of the column was then removed to let the flow start. The column was washed with 5 column volumes of 1X wash/binding buffer. A suspension of 1:1 Protein G agarose beads (Kirkegaard & Perry Laboratory, Gaithersburg, Maryland, USA) in 1X wash/binding buffer was prepared. The slurry was then poured into the column. The column was allowed to flow so that it was packed by gravitational pull. The packed affinity resin was equilibrated with 10 column volumes (CV) of wash/binding buffer.

4.2.6.7.2 ***Purification of mAb using Protein G column***

Ascites fluid was diluted in wash/binding buffer (1:1). A sample was taken for SDS-PAGE. Gently, the 1:1 suspension was applied to the column. The flowthrough was collected in a clean tube. A sample was taken for SDS-PAGE. The column was then washed with 10 CV of wash/binding buffer or until the Abs_{280nm} approached 0. Before elutiing, enough tubes (usually 6-8 tubes) were set aside, each containing 240 µl of 5X wash/binding buffer. To elute the antibody, the elution buffer was added 1ml at a time. Each 1 ml was collected in different tubes. Samples were taken from each tube (usually 10 µl) for SDS-PAGE gel analysis. Once the sample has been eluted, the affinity matrix was washed with 2 CV of elution buffer, followed by at least 10 CV

of 1X wash/binding buffer. After the equilibration of the column, the wash/binding buffer in the column was replaced with storage buffer and the column was kept in 4˚C.

4.2.6.7.3 *Preparation of dialysis tubing and dialysis*

The dialysis tubing was prepared according to the protocol described previously (Sambrook *et al*., 1989). Briefly, 10-20 cm long tubing was cut. The tubing was boiled for 10 minutes in large volume of 2% (w/v) sodium bicarbonate and 1 mM EDTA (pH 8.0). The tubing was then rinsed thoroughly in distilled water and boiled again in 1 mM EDTA (pH 8.0) for 10 minutes. The tubing was allowed to cool, then stored at 4˚C. Before use, the tubing was washed inside and out with distilled water.

Typically, protein solution was dialyzed in at least 500 times sample volume in suitable buffer overnight at 4ºC with 3 changes of buffer.

4.2.6.8 **Coupling of monoclonal antibody to Sepharose beads**

The Sepharose beads were treated according to the manufacturer's instructions. Briefly, 0.7 g (good for a 2 ml column) of CNBr-activated Sepharose 4B (Amersham Biosciences, Buckinghamshire, UK) was resuspended in 5 ml of 1.0mM HCl and allowed to swell by gentle shaking (3-5 minutes). Then the beads were washed with a total of 200 ml of 1 mM HCl using a column. After that, the beads were resuspended with 10 ml coupling buffer (0.1M $NaHCO_3$, 0.5M NaCl, pH8.3). The suspension was centrifuged at 2000 rpm (Jouan BR4i Centrifuge) for 5 minutes at 4˚C. The supernatant was discarded by aspirating. Then the beads were again resuspended in 5 ml coupling buffer and transferred to a 15 ml centrifuge tube. Around 1 mg of purified antibody was added to the suspension. Total volume of the suspension was adjusted using coupling buffer to make 10 ml final volume. The suspension was incubated

overnight at 4˚C with gentle shaking on Bio Dancer (New Brunswick Scientific, Edison, NJ, USA).

The next day, the suspension was centrifuged at 2000 rpm (Jouan Centrifuge) for 5 minutes at 4˚C. The supernatant was discarded and the beads resuspended with 4 ml blocking buffer (1 M ethanolamine, pH 8.0). The suspension was then incubated for 2 hours at room temperature (~25°C) with gentle shaking, after which the suspension was centrifuged again at 2000 rpm (Jouan Centrifuge) for 5 minutes at 4˚C to remove supernatant. A sample of the supernatant from each stage was collected to be analyzed using SDS-PAGE to check the coupling efficiency.

Finally, the beads were resuspended with 10 ml 1X TBS and pack in column or store at 4˚C for future use.

4.2.6.9 **Purification of native protein / yBlo t 19**

Previously packed column was washed with 1X TBS (pH 7.5) until OD_{280nm} = 0. 10 ml of *Blo t* mite crude extract (around 200-400 mg of protein) was then loaded to the column. Then, the column was washed with 10 CV of 1X TBS until OD_{280nm} approached 0. Elution with acidic condition was performed by adding 8 x 0.8 ml of acidic elution buffer (5mM Glycine (Sigma-Aldrich, Saint Louis, MO, USA), pH 2.7). Each 0.8 ml fraction was collected in 1.5 ml centrifuge tube containing 0.2 ml of 5X TBS pH 7.5 (for neutralizing the acidic condition).

After elution with acidic condition, the column was washed with 10 CV of 1X TBS. Wash until OD_{280nm} approached 0. Elution with basic condition was performed by adding 8 x 0.8 ml of basic elution buffer (5mM Glycine (Sigma-Aldrich, Saint Louis, MO, USA), pH 11) to the column. Each fraction was collected in 1.5 ml centrifuge tube containing 0.2 ml 5X TBS. Finally the column was washed with 20 CV of 1X TBS until OD_{280nm} approached 0. Then, the wash buffer in the column was

replaced with storage buffer (0.01 M NaH_2PO_4, 0.15 M NaCl, 2.7 mM KCl, pH 7.4, 20% ethanol), capped and kept in 4˚C.

300 µl from each fraction was collected for analysis. These samples were lyophilized and dissolved in 20 µl of sample buffer. Then, they were boiled and analyzed with SDS-PAGE (15% Tris-Glycine gel). After that, the protein was detected using silver staining.

Yeast expressed recombinant Blo t 19 was purified in similar manner.

4.3 *Results*

4.3.1 Blo t 19 sequence

Blo t 19 is a cysteine-rich protein with a deduced mature protein sequence of 66 residues (Figure 10). It has a theoretical molecular weight of 6792.67 daltons and isoelectric point (pI) value of 8.77 (calculated using Compute pI/Mw tool from Expaxy (http://tw.expasy.org/tools/pi_tool.html)).

```
                                                                    35
                                    gg  cca  gag  cac  ccc  gct  caa  gct  ctc  gac  ttt  acc
                                                      H    P    A    Q    A    L    D    F    T
                                                                                              95
agc tgt  gcc  cgg  atg  aac  gat  gga  gct  ctg  gga  gcc  aag  gta  gct  caa  gct  gcc  tgc  atc
S   C    A    R    M    N    D    G    A    L    G    A    K    V    A    Q    A    A    C    I
                                                                                             155
tcg  agt  tgc  aag  ttt  caa  aac  tgt  ggc  acg  gga  cac  tgt  gag  agg  cga  ggt  gga  cgt  cca
S    S    C    K    F    Q    N    C    G    T    G    H    C    E    R    R    G    G    R    P
                                                                                             215
acc  tgt  gtc  tgt  tct  cgc  tgt  ggc  aac  ggt  ggc  ggt  gaa  tgg  ccc  aat  ctg  ccc  tcc  agg
T    C    V    C    S    R    C    G    N    G    G    G    E    W    P    N    L    P    S    R
                                                                                             275
ggt  taa  tta  ttt  tgt  cat  ttg  gtc  tgt  gaa  agt  gtg  aca  cac  tat  tta  tat  atg  ctt  tca
G    -
                                                                                             335
ata  gga  ata  acc  ttc  aat  ttt  ggt  cac  act  ttc  cca  tca  att  ttt  gac  aat  ttc  aaa  att
                                                                                             395
aat  taa  ggt  ttt  att  tta  aag  ttc  atc  aat  ttt  ttg  tgg  ctt  tat  ttt  ttt  aaa  att  atc
                                                                                             455
cga  gat  gtt  tga  cag  aga  caa  ttg  att  aat  taa  aat  aat  ttt  tta  aat  tta  aaa  att  gtt
                                                                                             507
tta  taa  ata  aaa  att  ttt  tat  ttt  tgg  aaa  aaa  aaa  aaa  aaa  aaa  aat  tcg  t
```

Figure 10: Nucleotide sequence and the deduced amino acid sequence of Blo t 19. Number indicates the nucleotide position. The nucleotide sequence of the clone is 507bp in length. This includes a linker sequence ggccagag (blue), a 286bp 3' untranslated region with a poly-A tail, and a 218bp coding region for the recombination protein with a stop codon (TAA) at nucleotide residues 219-221. The inferred amino acid sequence from nucleotides 9 –218 indicated that this clone codes for a protein of 66 residues, with 8 cysteine residues (highlighted) in the molecule.

```
ASABF          GCAGTCGACTTTTCATCATGCGCACGTATG---GATGTACCTGGATTGAGCAAAGTGGCG
Blo t 19       GCTCTCGACTTTACCAGCTGTGCCCGGATGAACGATGGAGCTCTGGGAGCCAAGGTAGCT

ASABF          CAAGGATTATGCATATCTTCTTGCAAATTCCAGAATTGTGGTACCGGTCACTGTGAGAAG
Blo t 19       CAAGCTGCCTGCATCTCGAGTTGCAAGTTTCAAAACTGTGGCACGGGACACTGTGAGAGG

ASABF          CGTGGTGGTCGACCGACGTGTGTTTGCGATCGATGTGGACGAGGGGGCGGTGAATGGCCA
Blo t 19       CGAGGTGGACGTCCAACCTGTGTCTGTTCTCGCTGTGGCAACGGTGGCGGTGAATGGCCC

ASABF          AGCGTACCTATGCCAAAAGGGCGAAGTTCACGTGGACGAAGGCATTCTTA
Blo t 19       A----ATCTGCCCTCCAGGGGTTAA-------------------------
```

Figure 11: Alignment of Blo t 19 nucleotide sequences with ASABF nucleotide sequences encoding for the mature ASABF protein using ClustalW (http://clustalw.genome.ad.jp/). Matched sequences were highlighted (cyan).

```
ASABF          AVDFSSCARMDVPGL-SKVAQGLCISSCKFQNCGTGHCEKRG
Blo t 19       ALDFTSCARMNDGALGAKVAQAACISSCKFQNCGTGHCERRG

ASABF          GRPTCVCDRCGRGGGEWPSVPMPKGRSSRGRRHS
Blo t 19       GRPTCVCSRCGNGGGEWPNLPSRG----------
```

Figure 12: Alignment of Blo t 19 deduced amino acid sequence with ASABF mature protein sequence using Clustal W (http://clustalw.genome.ad.jp/). Matched sequences were highlighted (cyan). Matched cysteine residues were specially highlighted in yellow.

BLAST results revealed that Blo t 19 has high amino acid identity (70-76%) with *Ascaris suum* antibacterial factor (ASABF) (Accession number: BAA11943, Kato & Komatsu, 1996) and ASABF-family proteins, namely ASABF-beta (BAC00497), -gamma (BAC00498), -delta (BAC00499), and –zeta (BAC57992).

Blo t 19 aligned well with both the nucleotide and amino acid sequences of ASABF (Figure 11, Figure 12).

To deduced the possible structure of Blo t 19 based on information on ASABF, the cysteine array of Blo t 19 was compared with other Cysteine-Stabilized αβ (CSαβ) (Cornet *et al*., 1995)-type peptides as previously described (Zhang & Kato, 2003).

```
ASABF          ---MKTAIIVVLLVIFASTNAAVDFSSCARMDVPGL-SKVAQGLCISSCKFQN--CGTGHCE
Blo t 19       ----------------------ALDFTSCARMNDGALGAKVAQAACISSCKFQN--CGTGHCE
MGD-1          ------------------------GFGCPNNYQ-----------CHRHCKSIPGRCG-GYCG
Myticin A      ---MKATILLAVLVAVFVAGTEAHSHACTSYW------------CGKFCGTAS--CTHYLCR

ASABF          KRG-GRPTCVCDRCGRGGGEWPSVPMPKGRSSRGRRHS--------------
Blo t 19       RRG-GRPTCVCSRCGNGGGEWPNLPSRG------------------------
MGD-1          GW--HRLRCTCYRC--------------------------------------
Myticin A      VLH-PGKMCACVHCSRVNNPFRVNQVAKSINDLDYTPIMKSMENLDNGMDML
```

Figure 13: Comparison of cysteine array (highlighted) in Blo t 19 with other CSαβ-type peptides.

Cysteine array comparison showed that Blo t 19 shared conserved cysteine arrangements with other CSαβ-type peptides (Figure 13). This suggested Blo t 19 and ASABF having similar motif: CSαβ (Cornet *et al*., 1995). This motif is made of three distinct domains: an amino-terminal loop, a single α-helix and a two-stranded antiparellel β-sheet; the three of them were stabilized by disulfide bridges (Cornet *et al*., 1995).

4.3.2 Human IgE reactivity to Blo t 19

The importance of Blo t 19 human IgE reactivity was studied using plaque immunoassay and ELISA.

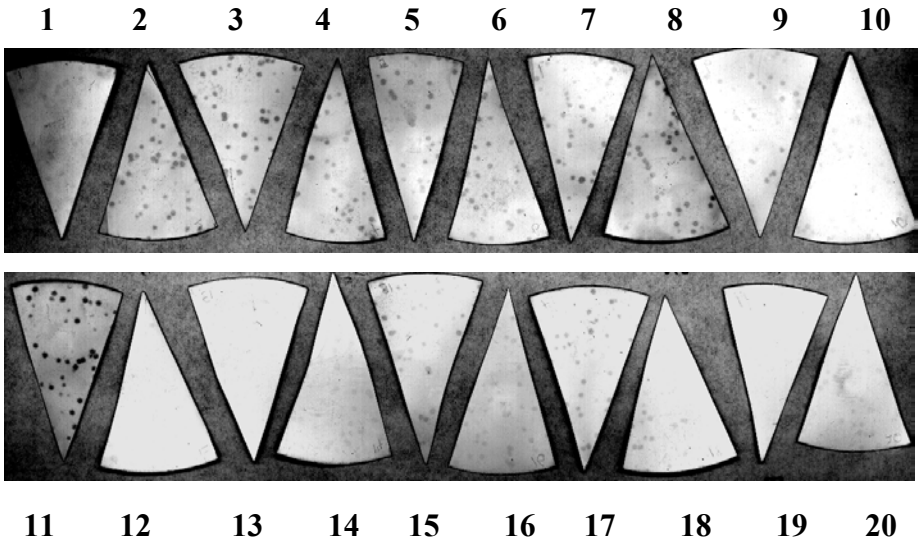

Figure 14: The plaque immunoassay showing the IgE reactivity of 20 sera tested with the recombinant protein Blo t 19. Panel number 2, 3, 4, 5, 6, 7, 8, 9, 11, 15, 16 and 17 are positive. Panel number 1, 14, 18 and 20 are slightly positive whereas panel number 10, 12, 13 and 19 are negative.

Plaque immunoassay showed that Blo t 19 was allergenic, binding IgE from 16/20 of the Blo t extract sensitized individuals (Figure 14).

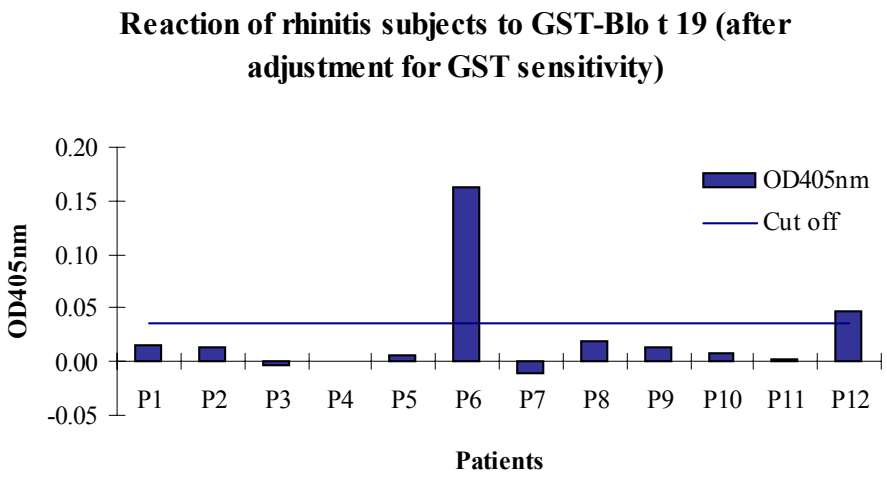

Figure 15: Allergenicity of GST-Blo t 19 among rhinitis subjects who were positive to Blo t extract. Cut off value was determined using mean plus two standard deviations of 10 healthy non-allergic subjects.

The Blo t 19 gene was expressed as a GST-fusion recombinant protein (GST-Blo t 19) by another colleague in the laboratory. Nonetheless, when GST-Blo t 19 was tested for its allergenicity using another panel of subjects, only 2/12 individuals reacted to GST-Blo t 19 (Figure 15).

4.3.3 Southern blot analysis

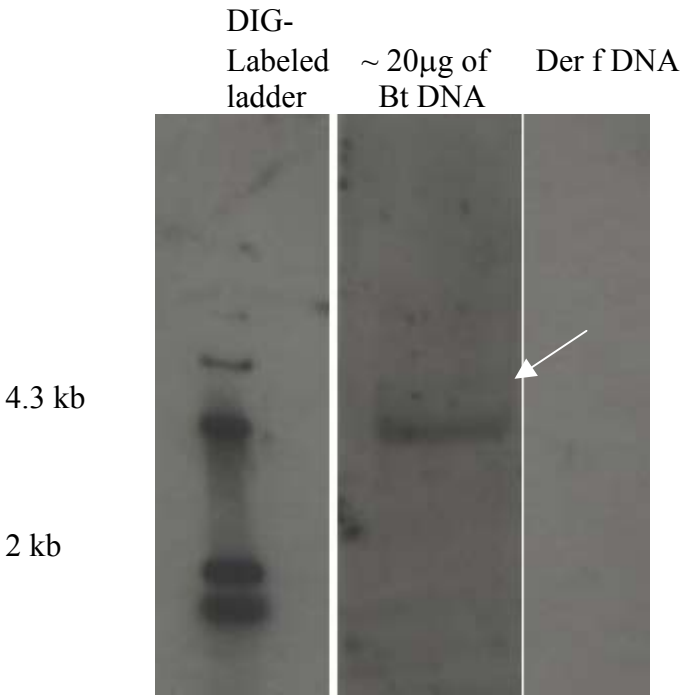

Figure 16: Detection of an around 4-.4.3 kb (white arrow) fragment in *Blo t* genomic DNA. *Der f* genomic DNA as negative control. All genomic DNA was digested with HindIII (Promega, Madison, USA).

Southern blot analysis revealed consistently a band of around 4-4.3 kb in the HindIII digested Blo t genomic DNA (Figure 16).

4.3.4 Monoclonal antibody generation

4.3.4.1 Cloning of Blo t 19 gene into pC1Derp5L expression vector

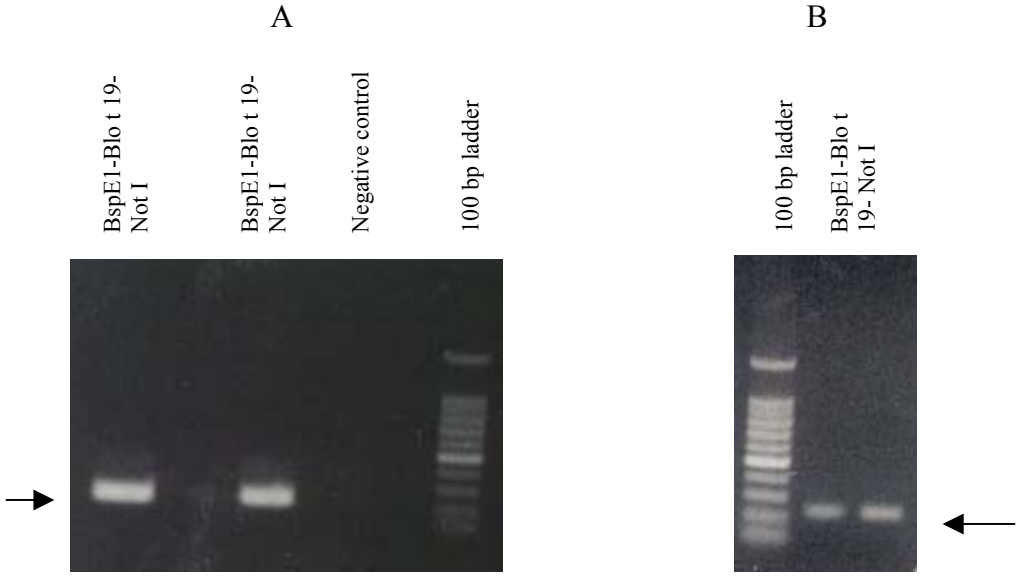

Figure 17: A: PCR using high fidelity polymerase to generate BspE1-Blo t 19-Not I gene fragment from pGEX-Blo t 19 clone; B: Purified PCR product.

Figure 17A showed the PCR product produced using pGEX-Blo t 19 as template, BspE1-Bt19 (Table 7) and Bt19-Not I (Table 7) as forward and reverse primers. Figure 17B showed the analysis of gel-purified PCR product (purified as previously described (4.2.2.5)). The purified BspE1-Blo t 19-Not I gene was then cloned into TOPO vector.

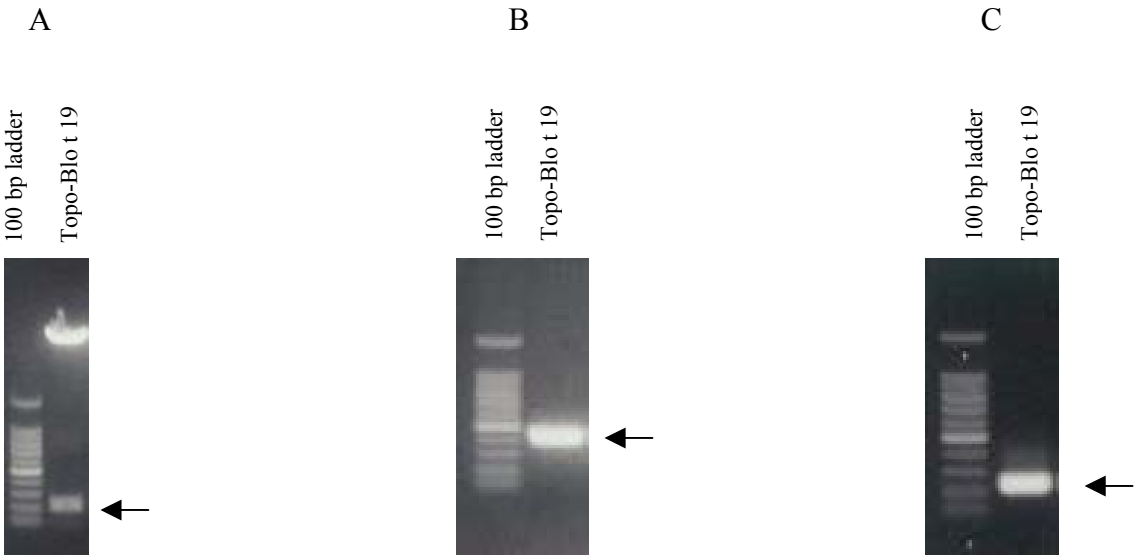

Figure 18: A: Restriction enzyme analysis of Blo t 19-TOPO clone; B: PCR product of Blo t 19 from Blo t 19-TOPO clone using M13 forward and reverse primers; C: PCR product using gene specific primers (BspE1-Blo t 19 and Blo t 19-Not I).

Figure 18A showed the result of restriction enzyme (RE) analysis of Blo t 19-TOPO clone. RE digestion released a fragment of ~200bp (identical to the one shown in Figure 17A-B) indicating positive result. Further analysis using PCR (Figure 18B-C) further proved that the clone was positive. Lastly, the clone was confirmed with sequencing (data not shown).

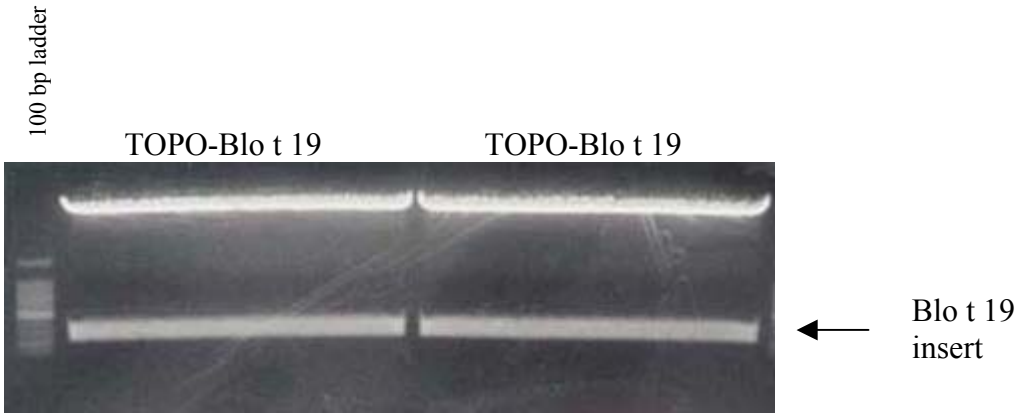

Figure 19: Preparation of BspE1-Bt19-Not I from TOPO-Bt19

Figure 19 showed the large-scale preparation of BspE1-Bt19-Not I gene fragment for cloning into pC1Dp5L vector (Figure 19). The Blo t 19 insert was gel-purified (4.2.2.5) and used for ligation.

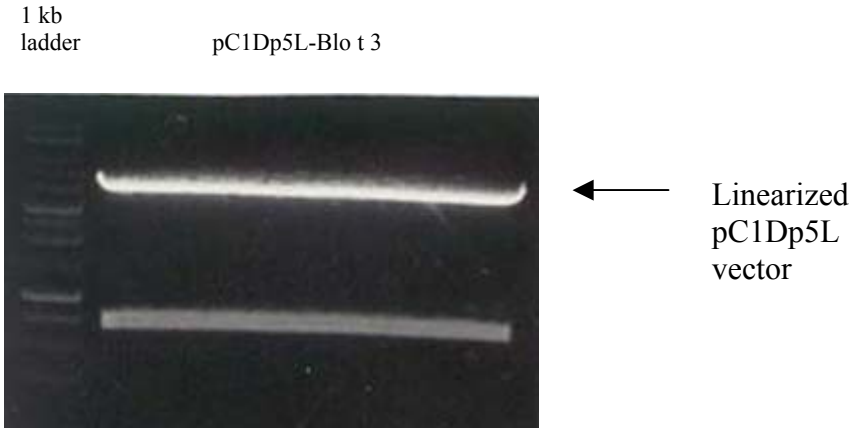

Figure 20: Preparation of BspE1-Not I linearized pC1Dp5L vector from pC1Dp5L-Blo t 3

Figure 20 showed the large-scale preparation of the pC1Dp5L vector (Figure 20). The BspE1-Not I linearized vector was gel-purified (4.2.2.5) and used for ligation.

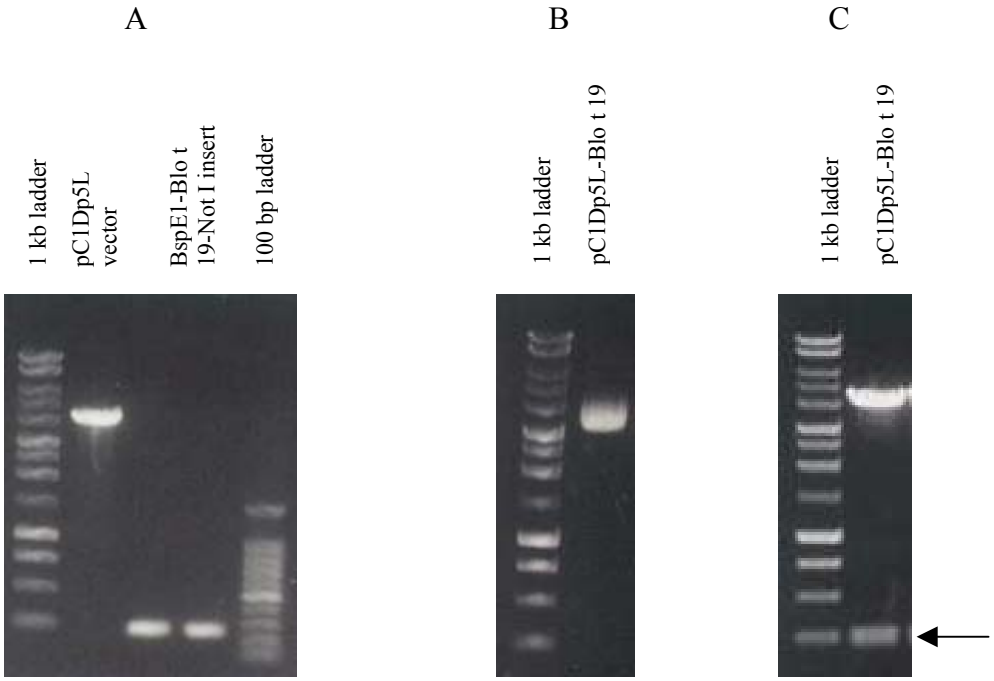

Figure 21: A: Gel purified linearized vector and insert; B: pC1Dp5L-Bt19 plasmid; C: Analysis of pC1Dp5L-Bt19 after treatment with BspE1 and Not I restriction enzymes.

Figure 21A showed the result of gel purification of both the vector and insert (Figure 21). Figure 21B-C showed the analysis of the pC1Dp5L-Bt19 clone using gel electrophoresis and RE digestion (Figure 21).

```
pC1Dp5L-Bt19    CTCCNNTCAATTCAGCTCTTAGGCTAGAGTCTTAATACGACTCACTATAGGCTAGCCTCT
Bt19FL          ------------------------------------------------------------

pC1Dp5L-Bt19    CGCCACCATGAAATTCATCATTGCTTTCTTTGTTGCCACTTTGGCAGTTATGACTGTTTC
Bt19FL          ------------------------------------------------------------

pC1Dp5L-Bt19    CGGAGCTCTCGACTTTACCAGCTGTGCCCGGATGAACGATGGAGCTCTGGGAGCCAAGGT
Bt19FL          ----GCTCTCGACTTTACCAGCTGTGCCCGGATGAACGATGGAGCTCTGGGAGCCAAGGT

pC1Dp5L-Bt19    AGCTCAAGCTGCCTGCATCTCGAGTTGCAAGTTTCAAAACTGTGGCACGGGACACTGTGA
Bt19FL          AGCTCAAGCTGCCTGCATCTCGAGTTGCAAGTTTCAAAACTGTGGCACGGGACACTGTGA

pC1Dp5L-Bt19    GAGGCGAGGTGGACGTCCAACCTGTGTCTGTTCTCGCTGTGGCAACGGTGGCGGTGAATG
Bt19FL          GAGGCGAGGTGGACGTCCAACCTGTGTCTGTTCTCGCTGTGGCAACGGTGGCGGTGAATG

pC1Dp5L-Bt19    GCCCAATCTGCCCTCCAGGGGTTAAGCGCATTCTTATAAGGGCGAATTCTGCAGATATCC
Bt19FL          GCCCAATCTGCCCTCCAGGGGTTAA----------------------------------

pC1Dp5L-Bt19    ATCACACTGGCGGCCGCTTCGAGCAGACATGATAAGATACATTGATGAGTTTGGACAAAC
Bt19FL          ------------------------------------------------------------
```

Figure 22: Alignment of one of the pC1Dp5L-Bt19 clones with Blo t 19 forward sequence. Matched sequences were highlighted (cyan).

Figure 22 showed the analysis of the sequencing result of pC1Dp5L-Bt19 (Figure 22). The result confirmed the clone was carrying Blo t 19 gene (Figure 22). Thus, pC1Dp5L-Bt19 plasmid was prepared in large scale and used for subsequent animal experiments.

4.3.4.2 Antibody responses obtained

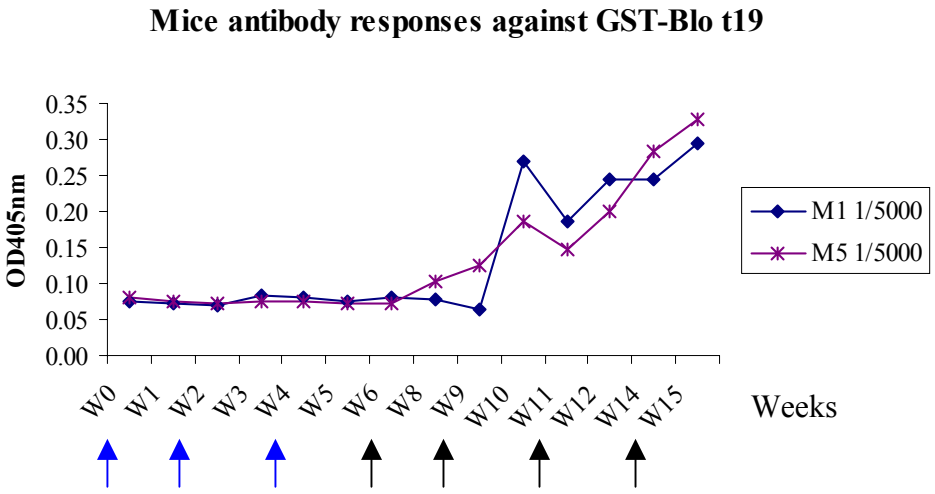

Figure 23: Mice antibody responses to GST-Blo t 19 after DNA immunization (blue arrow) and after protein boost with yeast-expressed Blo t 19 (black arrow). Mice did not react to Glutathione S-transferase (GST).

Figure 23 showed the immune responses of mice immunized with plasmid DNA encoding for Blo t 19 through i.m. and with electroporation. Antibody titre was very low after 3 doses of DNA immunization and electroporation (Figure 23). The mice had to be boosted with Blo t 19 expressed in *Pichia pastoris* (yBlo t 19) four times before fusion could be performed. The use of GST-Blo t 19 as screening reagent instead of yBlo t 19 was to show that both forms of protein could be used interchangeably as the mice that were immunized with yBlo t 19 could also react to GST-Blo t 19. The GST-form of Blo t 19 was preferred as it was purified whereas yBlo t 19 was from culture supernatant.

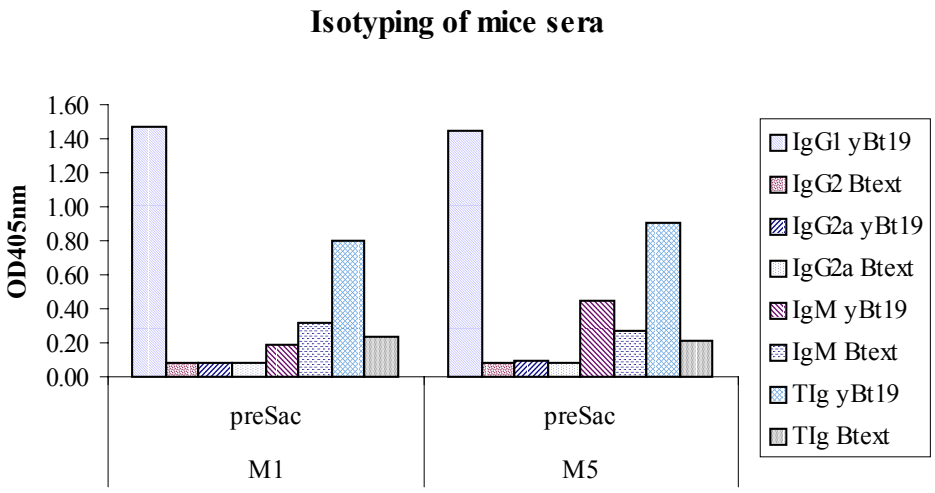

Figure 24: Isotyping of mice sera prior splenectomy. Mice sera were diluted 500 times. Note: Tig: Total antigen-specific immunoglobulins; yBt19: yBlo t 19

Isotyping of the sera prior fusion revealed a high IgG1 against yBlo t 19 and low IgG2a. The titer of IgM against yBlo t 19 was much lower compared to IgG1 (Figure 24) at the point the fusion was carried out.

Both intrasplenic delivery of DNA and s.c. delivery of Blo t 19 with CFA/IFA did not induce any detectable antigen specific antibody responses in the mice tested. Nevertheless, fusion was performed for mice treated with intrasplenic delivery of DNA.

4.3.4.3 Characterization of the monoclonal antibody produced

4.3.4.3.1 *Isotype of monoclonal antibody produced*

Isotypes	**Numbers**	**Remarks**
IgG1	1	AF6, good titre
Total	1	

Table 11: Blo t 19 specific monoclonal antibody generated from mice immunized by i.m. Blo t 19 DNA + electroporation + i.p. recombinant protein boost. A total of 9 hybridomas were obtained and screened.

Isotypes	**Numbers**	**Remarks**
IgG2a	1	Very low titre (I3D3)
IgM	10	
IgA	2	Very low titre
Total	13	

Table 12: Monoclonal antibody generated from mice immunized by i.m. Blo t 19 DNA + electroporation + i.s. injection of Blo t 19 DNA. A total of 301 hybridomas were obtained and screened.

Isotypes	**Numbers**	**Remarks**
IgM	6	Reacted against Blo t extract only
Total	6	

Table 13: Monoclonal antibody generated from mice immunized by i.s. injection of Blo t 19 DNA alone. A total of 28 hybridomas were obtained and screened.

4.3.4.3.2 ***Specificity of Bt19 monoclonal antibody (AF6)***

4.3.4.3.2.1 **ELISA**

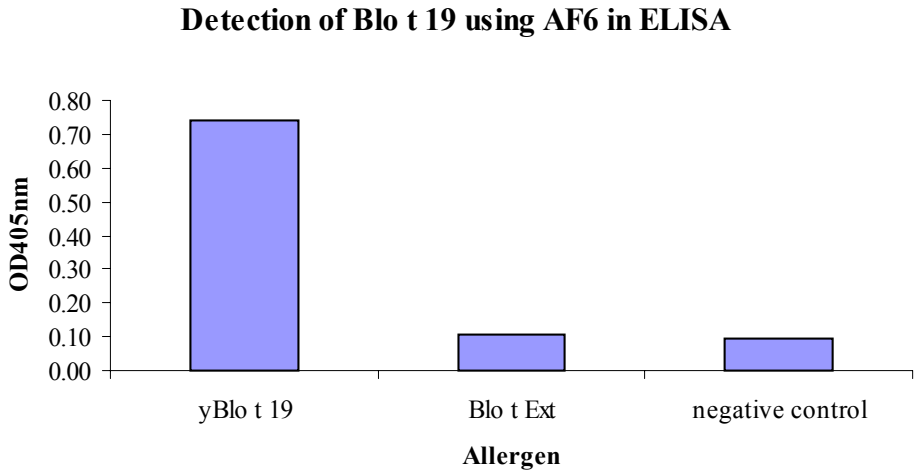

Figure 25: Screening of AF6 hybridoma supernatants using yBlo t 19 and Blo t extracts. Negative control was cell culture medium alone without antibody. The antibody was detected using rat anti-mouse IgG1 biotin conjugated (1:2000).

Figure 25 showed the screening results of AF6 hybridoma supernatants using yBlo t 19 and Blo t extracts (Figure 25). Negative control was cell culture medium alone without antibody. The antibody was detected using rat anti-mouse IgG1 biotin conjugated (1 : 2000) (Figure 25).

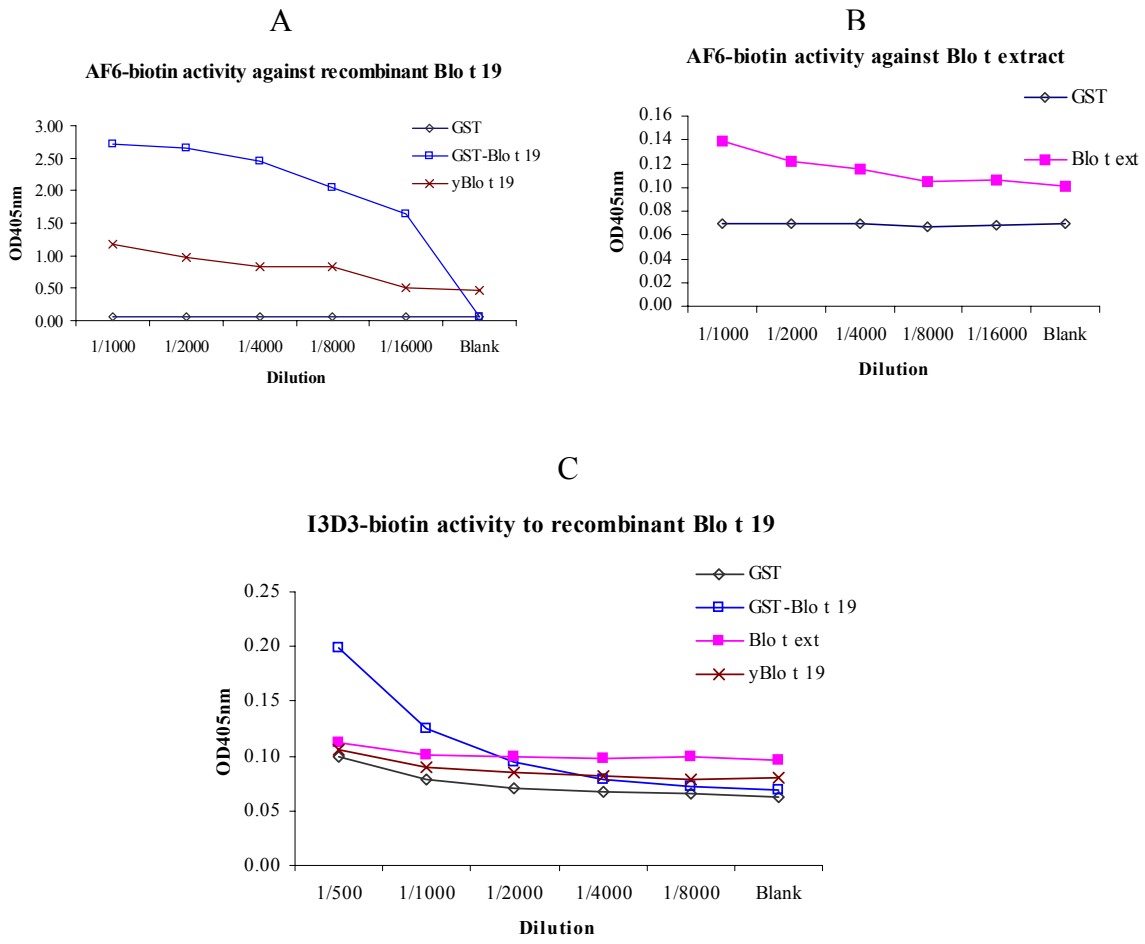

Figure 26: Activity of different dilutions of biotinylated AF6 and I3D3 against recombinant Blo t 19 and Blo t extract. Note: GST was negative control. Blank was wells without antibody.

Biotinylated AF6 reacted with GST-Blo t 19 and yBlo t 19, with lower reactivity with the later (Figure 26A). However, it reacted slightly with Blo t extract and negative to GST recombinat protein (Figure 26B). As I3D3 almost did not react with *Blo t* mite extract and yBlo t 19 (Figure 26C). No further work was performed using I3D3.

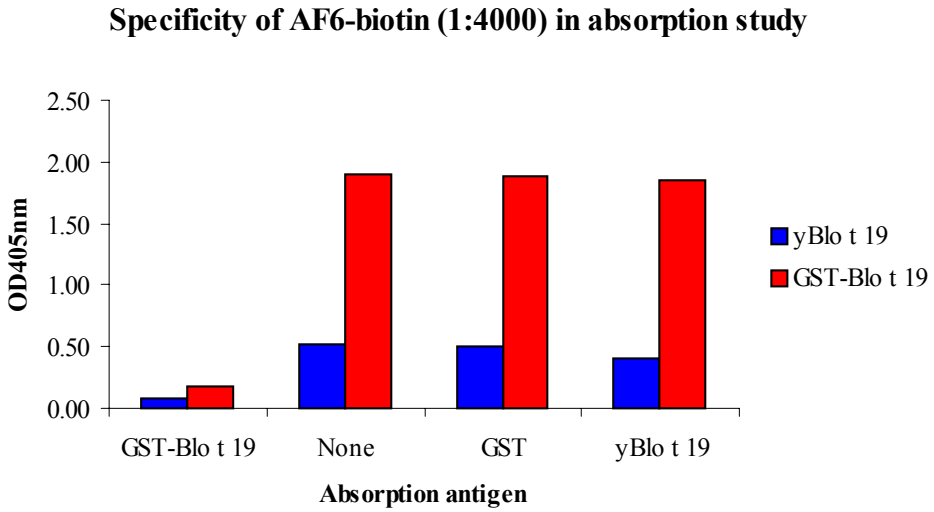

Figure 27: Specificity of AF6 mAb to GST-Blo t 19 through absorption study. Antigens listed on x-axis were used to absorb AF6-biotin and tested against antigens: yBlo t 19 (blue) and GST-Blo t 19 (red).

As shown in Figure 27, AF6 was very specific against GST-Blo t 19. It did not react to GST at all. Its binding to yBlo t 19 was weak compared to GST-Blo t 19 as shown by the slight reduction after absorption (Figure 27).

4.3.4.3.2.2 **Western Blot**

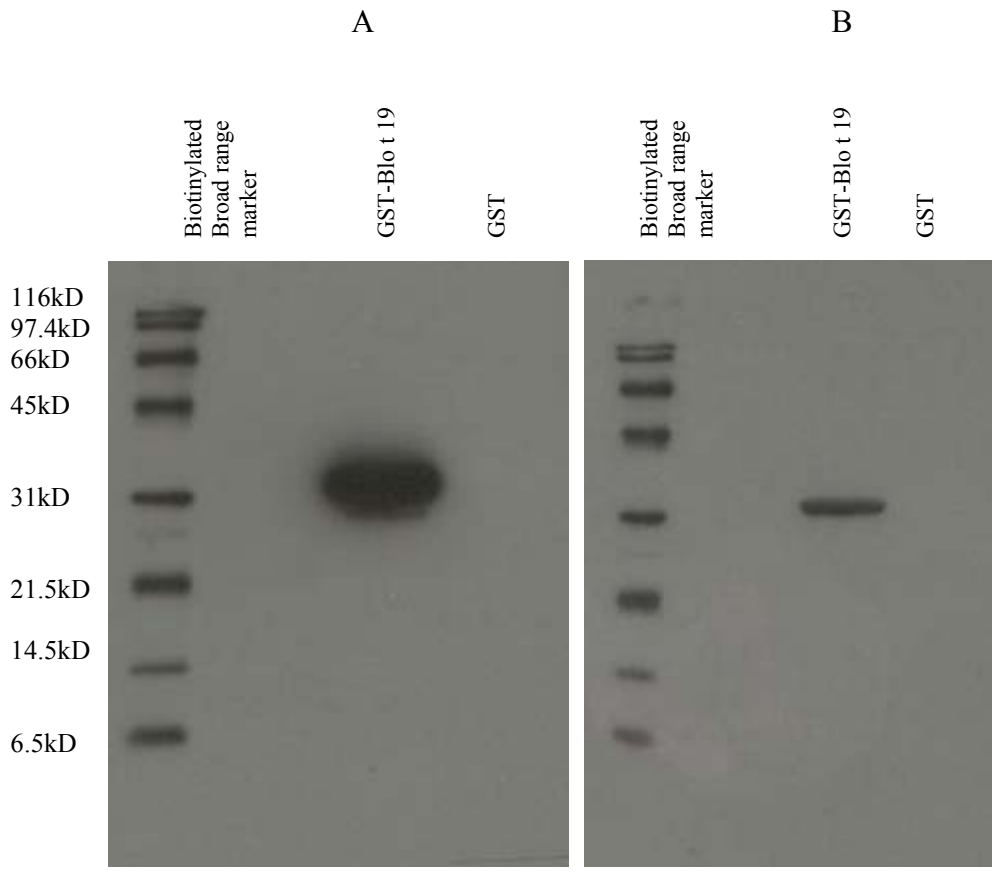

Figure 28: A: Western blot result of antibody AF6 without prior incubation with 20 μg of GST-Blo t 19; B: with prior incubation of 20 μg of GST-Blo t 19. GST: Glutathione S-transferase. Each lane was loaded with 0.5 μg of protein.

Figure 28 further showed the specificity of AF6 to GST-Blo t 19 in western blot (Figure 28).

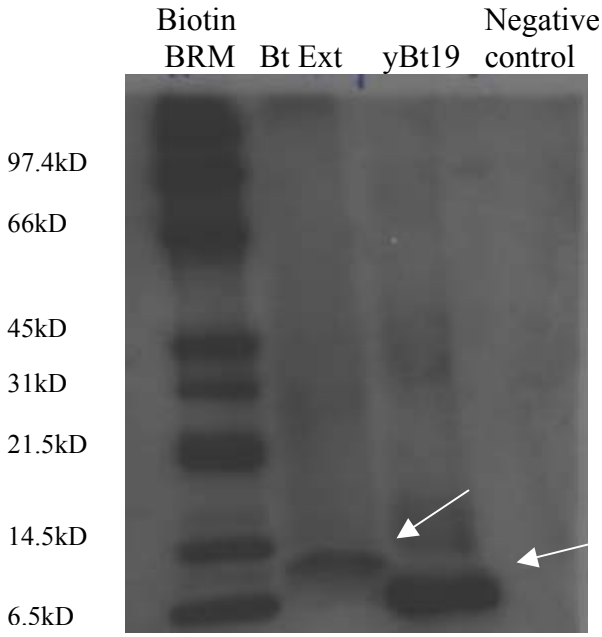

Figure 29: Detection of Blo t 19 in mite extract yBlo t 19 (white arrows) using western blot by AF6 (1:1000). Negative control was another unrelated yeast expressed *Blo t* allergen.

AF6 could be used to detect Blo t 19 in *Blo t* mite extract as well as yBlo t 19 in western blot (Figure 29).

4.3.4.4 **Purification of monoclonal antibody from ascites fluid**

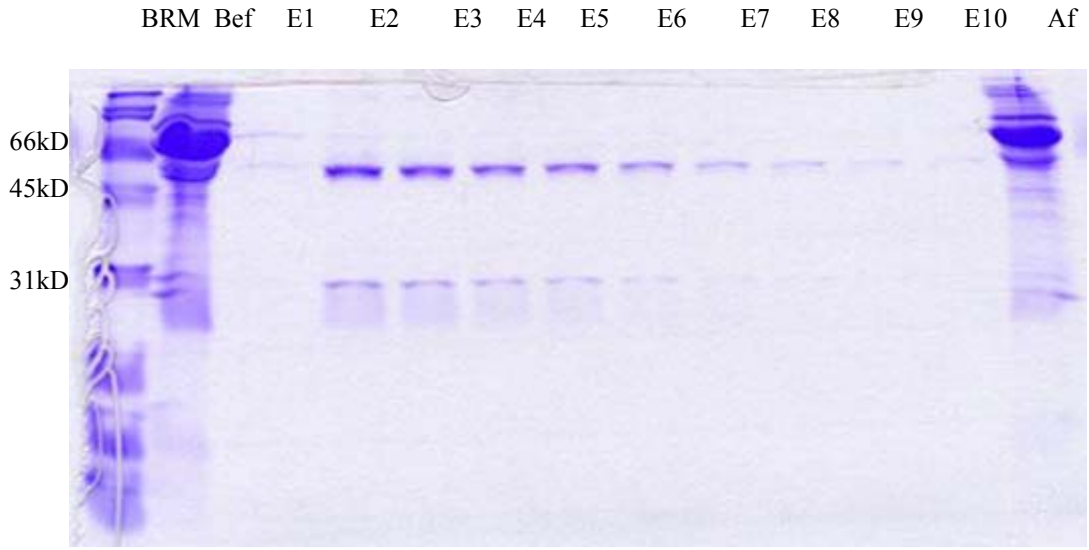

Figure 30: Purification of AF6 from ascites. BRM: Broad Range Marker (Bio-Rad) (the relevant sizes marked); E1-E10: samples from eluted fractions. Bef: ascites before purification; Af: ascites after going through the column.

Purified AF6 antibodies were pooled together (E2 to E9) (Figure 30) for dialysis and coupled to Sepharose beads or for biotinylation.

4.3.4.5 **Purification of native Blo t 19**

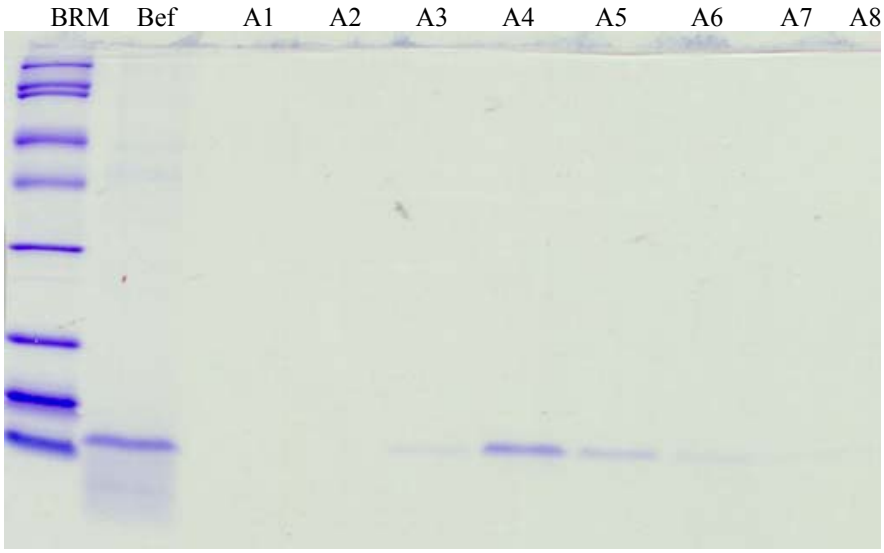

Figure 31: Purification of *Pichia pastoris* expressed yBlo t19 using AF6 mAb immunoaffinity column --- a proof of concept. BRM: Broad range marker (Bio-Rad); A1-A8: samples from eluted fractions using acidic elution buffer. Bef: sample before purification.

AF6 immunoaffinity column was proven to be able to purify Blo t 19 expressed in *Pichia pastoris* (Figure 31).

However, isolation of native Blo t 19 from the crude mite extract was not successful when the same AF6 monoclonal antibody immunoaffinity column was used (data not shown).

4.3.4.6 Mouse strain difference study

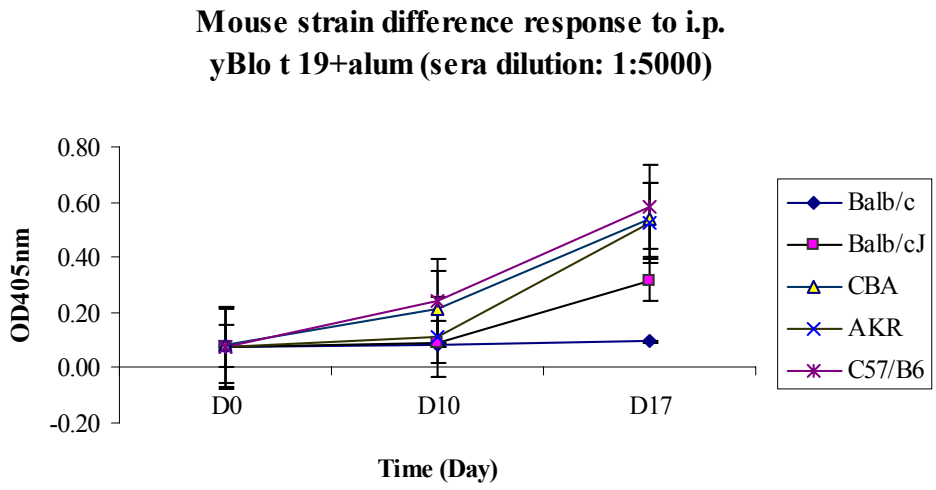

Figure 32: Antibody responses (total antigen-specific immunoglobulins) between mouse strains in response to i.p. injection of alum-coupled yBlo t 19. Each data point represented average readings of 3 mice.

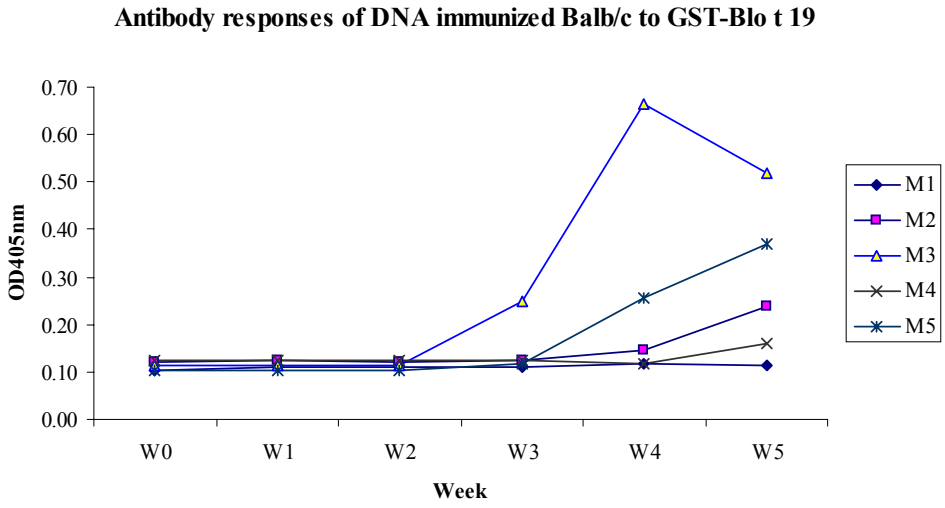

Figure 33: Antibody responses of DNA immunized Balb/c mice to GST-Blo t 19. Mice sera were diluted 250 times. Mice sera did not react to GST.

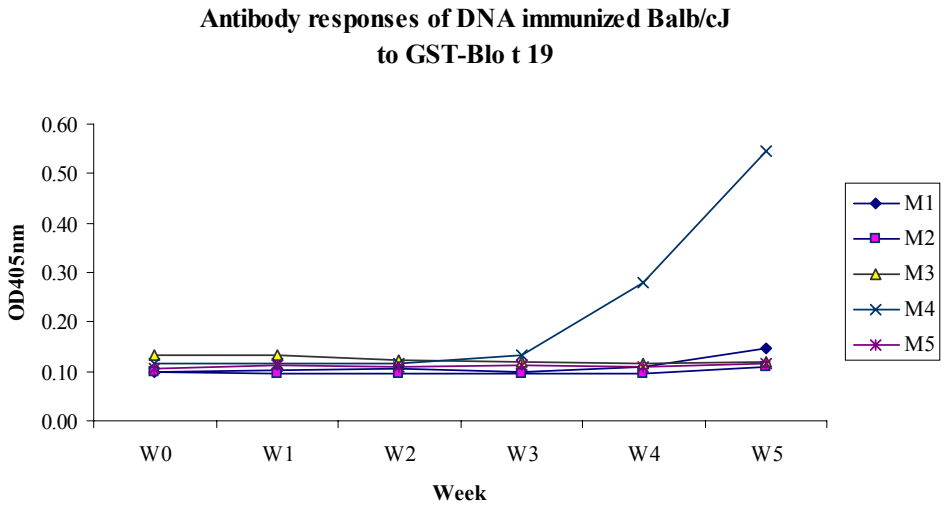

Figure 34: Antibody responses of DNA immunized Balb/cJ to GST-Blo t 19. Mice sera were diluted 250 times. Mice sera did not react to GST.

Mice from different strains reacted differently to yeast recombinant Blo t 19 delivered via i.p. with alum (Figure 32). C57/B6, CBA and AKR responded well to this immunization protocol and gave high antibody responses. On the other hand, Balb/c and Balb/cJ which were usually used for monoclonal antibody generation experiments (Westerwoudt *et al*., 1984; Hong *et al*., 1989; Kilpatrick *et al*., 1997; Moonsom *et al*., 2001; Kasinrerk *et al.*, 2002; Yang *et al*., 2003; Ramos *et al*., 2003) reacted poorly to the protocol.

In terms of DNA immunization, Balb/c mice seem to respond better compared to Balb/cJ with respect to their antibody responses to GST-Blo t 19 (Figure 33, Figure 34). Although both strains had low antibody titer against GST-Blo t 19, more Balb/c mice had a better antibody titer to GST-Blo t 19 than Balb/cJ. This was in contrast to the antibody responses obtained via i.p. delivery of protein with alum (Figure 32) where Balb/cJ responded better than Balb/c.

4.4 *Discussion*

4.4.1 Unique *Blo t* allergen, Blo t 19

A unique *Blo t* allergen, designated Blo t 19, was identified via screening of of λgt11 expression library of *Blomia tropicalis*. Blo t 19 shared 76% sequence identity with ASABF. The good alignment of Blo t 19 with both the nucleotide and amino acid sequences of the mature ASABF suggested that both proteins could potentially be evolutionarily linked. Blo t 19 is the first protein beyond nematodes that has high sequence similarity with ASABF.

ASABF has been identified as a CSαβ-type antimicrobial peptide by ^{1}H-NMR (Zhang & Kato, 2003). It was known that all CSαβ-type antimicrobial peptides had conserved cysteine array to give them their characteristic structure: a single α-helix and a pair of anti-parellel β-sheets (Bontems *et al*., 1991; Cornet *et al*., 1995; Dimarcq *et al*., 1998). ASABF could be considered distantly related to insect defensins (Dimarcq *et al*., 1998). Previous study showed that the cysteine array of ASABF was more identical to MGD-1 and myticin A, both antimicrobial peptides from two species of mussels: *Mytilus galloprovincialis* and *Mytilus edulis*, respectively than to other defensins based on cysteine array comparison (Zhang & Kato, 2003). It was demonstrated in this study that Blo t 19 had the same cysteine array as well (Figure 13), suggesting that Blo t 19 could have similar structure as these proteins.

In terms of allergenicity, Blo t 19 did not seem to be a major allergen as far as the recombinant forms are concerned. Only around 10% of the subjects reacted to GST-Blo t 19 in ELISA. Nonetheless, it bound IgE in 16/20 sera from mite sensitized subjects in plaque immunoassay (Figure 14). One possible explanation was that GST-Blo t 19 was not properly folded. The fact that Blo t 19 was relatively small compared to GST, it was very likely that the epitopes were masked by GST. To address this

discrepancy, native Blo t 19 is required. Therefore, effort was made to raise monoclonal antibody against this allergen with the hope that the native form of this allergen could be isolated.

4.4.2 Monoclonal antibody generation

The idea of using DNA immunization to induce antibody in mice for monoclonal antibody production experiments was based on similar experiments in the laboratory (Ramos *et al*., 2003; Wolfowicz *et al*., 2003; Yang *et al*., 2003). Monoclonal antibodies specific to Blo t 3 and Blo t 11 were successfully produced through DNA immunization (Ramos *et al*., 2003; Yang *et al*., 2003).

4.4.2.1 **Applications of AF6 (Blo t 19-specific mAb)**

A useful monoclonal antibody (AF6) specific for recombinant Blo t 19 was successfully raised. There was no question about the specificity of the antibody towards GST-Blo t 19 as shown by the specific inhibition of binding in ELISA (Figure 27). Although absorption study with yBlo t 19 only reduced slightly in the binding of AF6 to GST-Blo t 19 (Figure 27), the fact that yBlo t 19 could be purified by using AF6 immunoaffinity column and also detected in ELISA showed that AF6 actually bound yBlo t 19.

It was quite clear that AF6 could only give good signals in western blot when used to detect Blo t 19 in mite extract (Figure 29). This was not uncommon as other investigators also reported similar observations with other monoclonal antibody where it was shown that certain antibody was good for western blot but not for indirect fluorescence (Chestukhin & DeCaprio, 2003). It was possible that AF6 recognized linear epitope exposed only when Blo t 19 was denatured or misfolded. AF6 could immunopurify yBlo t 19 but not native Blo t 19 because yBlo t 19 might exist in a

misfolded form which favored the binding of AF6 whereas the native form of Blo t 19 might have a different conformation.

It was observed that both AF6 and I3D3 actually recognized GST-Blo t 19 better than yBlo t 19 (Figure 26) albeit the mice were not exposed to GST-Blo t 19 at all during immunizations. On top of that, the hybridomas were screened using yBlo t 19 and *Blo t* mite extract (Figure 25). This could be due to the small size of Blo t 19. The epitope of the yeast-expressed form probably could have been hidden or misfolded when coated onto the ELISA plates or transferred to membrane in western blot. In the case of GST-Blo t 19, because the size of GST was much larger than Blo t 19, the chances that it was absorbed to the plates and membranes was higher thus leaving Blo t 19 free to interact with the antibody. This may explain the difference in the intensity of optical density reading in ELISA between GST-Blo t 19 and yBlo t 19 under the same concentration (Figure 27).

4.4.2.2 **Factors affecting the success of monoclonal antibody generation**

The shortcomings of this study were that it did not manage to raise more Blo t 19 specific monoclonal antibodies and the screening process. More monoclonal antibodies would allow for more choices in choosing the right antibody for immunopurifications. The screening process involved recombinant proteins as antigens although the immunization processes mainly involved DNA delivery. Considering this issue, any antibody recognizing conformational epitope produced by the hybridomas could have escaped detection if the folding of the protein expressed *in vivo* in mice was different from those expressed in *E. coli* or *Pichia pastoris*. Moreover, initial DNA immunization did not raise significant amount of Blo t 19 specific antibodies in the mice (Figure 23). The antibody titer rose only after i.p. delivery of alum-coupled yBlo t 19. Therefore, the production of antibodies could have been induced by alum-coupled

yBlo t 19 alone. Isotyping of the mice sera collected prior sacrifice showed that this was indeed the case (Figure 24). The mice sera indeed showed a high IgG1 titer compared to IgG2a, suggesting a Th2 response (Figure 24) (Raz *et al*., 1996). Gene immunization was known to induce primarily Th1 response which was signified by high titer of IgG2a. On the other hand, protein immunization induced Th2 response, with high IgG1 and often IgE production (Raz *et al*., 1996). In addition, it was known that protein immunization using recombinant proteins does not always produce antibodies that recognize native epitopes (Attanasio *et al*., 1997). Therefore, another fusion was performed using purely DNA immunization and I3D3 was obtained as a result. Nonetheless, the titer and affinity of I3D3 was too low to be of any use.

Besides that, greater number of hybridomas generated did not favor the increasing chance of getting IgG-isotype antigen-specifc monoclonal antibody producing hybridomas. This observation was also obtained in similar study using another allergen, Blo t 12, where although hundreds of hybridomas were obtained, none was producing IgG-isotype antigen-specific monoclonal antibodies (unpublished data).

It was observed that DNA immunization indeed induced lower titers of antibody compared to protein immunization as previously reported (Attanasio *et al*., 1997). In fact, the antibody level induced by DNA immunization in this study was almost undetectable. Nevertheless, IgG-isotype monoclonal antibody (I3D3) could also be produced through DNA immunization. The only setback was that its titer and affinity was too low to be of any use.

Different genetic background gave different degree of immune response to the same antigen (Berzofsky *et al*., 1977; Chatel *et al*., 2003). This was also demonstrated in this study (Figure 32-Figure 34). It appeared in this study that Balb/c mice were

more responsive towards DNA immunization compared to Balb/cJ although Balb/cJ gave better response in protein immunization (Figure 33, Figure 34). Nonetheless, due to the small number of animals in each group (3-5 per group), no conclusive statement could be made. Further experiments were required to verify this observation.

4.5 *Conclusions and future directions*

In conclusion, a novel *Blo t* allergen, Blo t 19 has been identified. It is a probable antimicrobial peptide which has high amino acid sequence identity with ASABF, an antimicrobial peptide from *Ascaris suum*.

Recombinant Blo t 19 is a minor allergen. The allergenicity of native Blo t 19 remains to be elucidated, as it has not been obtained successfully from the crude extract using monoclonal antibody column in this study. Further optimization of the experimental protocol is necessary in order to obtain the native Blo t 19. Nonetheless, the monoclonal antibody raised is useful in detecting Blo t 19 in western blot and ELISA.

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Appendices

Appendix A: Reagents

Acidic elution buffer (5 mM Glycine, pH 2.7)

1 M Glycine (Sigma*)	0.5 ml
Milli-Q water	50 ml

Adjust to pH 2.7
Top up to 100 ml with Milli-Q water.

Agarose gel (1%)

Seakem® LE agarose (BioWhittaker*)	4.0 g
1 x TAE Buffer	400 ml

microwave mixture, add ethidium bromide (BioRad*) (final concentration=0.5 µg/mL).
Store in 50°C oven. Pour on tray when required.

Alum

Amphojel Antacid Suspension (Whitehall*)	2 mg

10% (w/v) Ammonium Persulfate (10% APS)

Ammonium persulfate (Amresco*)	1.0 g

Top up to 10 ml with Milli-Q water.
Mix well, store at 4°C.

Ampicillin, **100 mg/ml**

Ampicillin, sodium salt (Calbiochem*)	5.0 g

Top up with Milli-Q water to 50 ml, mix well, filter sterilize. Store in aliquots of 1 ml / tube at -20°C.

Basic elution buffer (5mM Glycine, pH 11.0)

1 M Glycine (Sigma*)	0.5 ml
Milli-Q water	50 ml

Adjust to pH 11.0
Top up to 100 ml with Milli-Q water.

500x Biotin (0.02%)

Biotin (Sigma*)	0.02%
add Milli-Q water and filter sterilize.	(v/v)

Biotinylation reagent

Biotinamidocaproate N-hydroxysuccinimide ester (Sigma*)	1 mg
Dimethylsulfoxide (DMSO) (Sigma*)	1 ml

Mix well and store in 4°C in aliquots.

Buffered Glycerol-complex (BMGY) Medium (per Liter)	10 g
Yeast extract (Becton*)	20 g
Peptone (Becton*)	100 ml
dissolve in 700 mL water and autoclave. Allow to cool and add:	100 ml
1 M potassium phosphate, pH 6.0 (Sigma*)	2 ml
10x Yeast Nitrogen Base (YNB) (Becton*)	100 ml
500x Biotin (Sigma*)	
10x glycerol (BDH*)	

Buffered Glycerol-complex (BMMY) Medium (per Liter)	
Yeast extract (Becton*)	10 g
Peptone (Becton*)	20 g
dissolve in 700 mL Milli-Q water and autoclave.	
Allow to cool and add:	
1 M potassium phosphate, pH 6.0 (Sigma*)	100 ml
10x YNB (Becton*)	100 ml
500x Biotin (Sigma*)	2 ml
10x Methanol (Merck*)	100 ml
To prepare Buffered Methanol-complex (BMGY) Medium, the Methanol was replaced with 10x glycerol (BDH*).	

Coomassie stain	
Coomassie Brilliant Blue R-250 (BioRad*)	1 g
Absolute ethanol (Sino*)	300 ml
Glacial acetic acid (Merck*)	100 ml
Stir overnight to dissolve the coomassie, top up to 1 litre with Milli-Q water.	

Coupling Buffer	
$NaHCO_3$ (Sigma*)	8.401 g
5 M NaCl	100 ml
Milli-Q water	700 ml
*Adjust to pH 8.3	
Top up to 1 litre with Milli-Q water.	

CRC cocktail	
Hypnorm® (Janssen*)	1 ml
Dormicum® (Roche*)	1 ml
Milli-Q water (sterile)	2 ml

Destaining solution (for Coomassie staining)	
Absolute ethanol (Sino*)	300 ml
Glacial acetic acid (Merck*)	50 ml
Top up with Milli-Q water to 1 litre.	

Detection Buffer	
Tris base (JT Baker*)	12.114 g
NaCl (Merck*)	5.844 g
Milli-Q water	700 ml
Adjust pH to 9.5 and top up to 1 litre with Milli-Q water.	

Developer solution (for silver staining)
$NaHCO_3$ (Sigma*) 20 g
Top up to 1 litre with Milli-Q water.
Add formalin (Sigma*) to final concentration of 0.04% (v/v) prior use.

10x Dextrose (20%)
D-glucose (Sigma*) 200 g
Add water to make 1 L and filter sterilized.

1 M Dithiothreitol (DTT)
Dithiothreiol (BioRad*) 3.09 g
Sodium acetate (pH 5.2) 20 ml
Sterilize by filtration, aliquot in 1 ml and store at -20°C.

0.5 M EDTA (pH 8.0)
Disodium ethylenediaminetetraacetate ($Na_2EDTA.2H_2O$) (BioRad*) 186.1 g
Milli-Q water 800 ml
Stir vigorously on a magnetic stirrer and adjust to pH 8.0 with around 20 g of NaOH pellets. EDTA will not dissolve completely until pH 8.0. Aliquot and sterilize by autoclaving.

Extration buffer 1 (For sensitization profile study)
10X PBS 10 ml
0.5 M EDTA 200 µl
0.25 M PMSF 800 µl
Top up to 100 ml with Milli-Q water.

Extration buffer 2 (For purification study)
1X TBS 40 ml
0.5 M EDTA 100 µl
0.25 M PMSF 400 µl
Top up to 50 ml with 1X TBS and store at 4°C. Prepare prior use.

Fixing solution (for silver staining)
Methanol (Mallinckrodt*) 500 ml
Glacial acetic acid (Merck*) 50 ml
Top up to 1 litre with Milli-Q water.

1 M Glycine
Glycine (Sigma*) 7.508 g
Top up to 100 ml with Milli-Q water. Sterilized by autoclaving.

0.2 M Glycine (Elution buffer)
1 M Glycine 100 ml
Milli-Q water 200 ml
Adjust pH to pH 2.85
Top up to 1 liter with Milli-Q water. Filter sterilized and store at 4°C.

HT medium

DMEM (Dulbecco's Modified Eagle's Medium 1X), HyClone*	450 ml
Fetal Bovine Serum, HyClone*	50 ml
HyQ Penicilin-Streptomyocin solution, HyClone*	5 ml
L-Glutamine, HyClone*	5 ml
HyQ Sodium Pyruvate, HyClone*	5 ml
HT supplement, Gibco*	5 ml

Isopropylthio-β-D-galactoside (IPTG)

IPTG	2 g
Milli-Q water	8 ml

Dissolve and adjust volume to 10 ml. Filter sterilized, store in -20ºC in 1 ml aliquots.

LB Broth

Luria Bertani Broth (Becton*)	25.0 g

Top up to 1 litre with Milli-Q water. Sterilize by autoclaving and store at 4°C. Warm up to room temperature prior use.

LB Agar with Ampicillin

Luria Bertani broth (Becton*)	25.0 g
Agar (Becton*)	20.0 g

Top up to 1 litre with Milli-Q water and autoclave in liquid cycle.
1 ml Ampicillin 100 mg/ml, added when agar cool to around 50°C.
Mix well and pour into plate immediately. Store plates at 4ºC.

Maleic acid buffer

Maleic acid (BDH*)	13.4 g
NaCl (Merck*)	8.766 g
Milli-Q water	600 ml

Adjust pH to 7.5 with NaOH pellet (Merck*) and top up to 1 litre with Milli-Q water.
Washing buffer was prepared by adding Tween 20 to Maleic acid buffer to a final concentration of 0.3% (v/v).

5 M NaCl

NaCl (Merck*)	292.2 g
Milli-Q water	700 ml

Dissolve and top up to 1 litre with Milli-Q water. Sterilize by autoclaving.

NZY Agar

NaCl (Merck*)	5 g
MgSO4 7H2O (Sigma*)	2 g
Yeast extract (Becton*)	5 g
Casein Hydrolysate (Becton*)	10 g
Bacto Agar (Becton*)	15 g

Top up to a final volume of 1 litre with Milli-Q water. Adjust pH to 7.5 with NaOH (Merck), autoclave, and pour ~80 mL/145 mm plate.

10X Phosphate buffered saline (10X PBS)

NaCl (Merck*)	80 g
KCl (Merck*)	2 g
$Na_2HPO_4.12H_2O$, (Merck*)	36.3 g
KH_2PO_4 (Merck*)	2.4 g

Adjust pH to pH 7.4
Top up to 1 litre with Milli-Q water

PBS-T

10X PBS	100 ml
Tween polysorbate 20 (Duchefa*)	0.500 ml

Top up to 1 litre with Milli-Q water

0.25 M PMSF

Phenylmethyl-sulfonyl fluoride (PMSF) (Sigma*)	0.871 g
Absolute alcohol (Ethanol) (Hayman*)	20 ml

Dissolve well, aliquot and store at -20°C

10% SDS

Sodium dodecyl sulfate / Sodium lauryl sulfate	100 g
Milli-Q water	900 ml

Heat to 68°C to assist dissolution, adjust to pH 7.2. Adjust volume to 1 litre, dispense into aliquots

2X SDS-PAGE Loading dye

Milli-Q water	1.8 ml
1 M Tris-HCl pH 6.8	1.2 ml
Glycerol (BDH*)	2.0 ml
10% (w/v) SDS	4.0 ml
0.2% (w/v) Bromophenol Blue	0.5 ml
200 mM DTT	0.5 ml

Aliquot and store at 4ºC

Sensitizing solution (for silver staining)

$Na_2S_2O_3$ (Merck*)	0.2 g

Top up to 1 litre with Milli-Q water

0.1% (w/v) Silver Nitrate Solution

Silver nitrate (Merck*)	1.0 g

Top up to 1 litre with Milli-Q water and keep in the dark at 4°C

Sodium deoxycholate 51.3 mg/ml

Deoxycholic acid, sodium salt (Sigma*)	5.13 g

Top up to 100 ml with Milli-Q water.

Stop solution (for silver staining)

$Na_2EDTA.2H_2O$ (BioRad*)	14.6 g

Top up to 1 litre with Milli-Q water.

50X TAE Buffer (Tris-acetae/EDTA electrophoresis buffer)

Tris base (JT Baker*)	242 g
Glacial acetic acid (Merck*)	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml
Top up to 1 litre with Milli-Q water	

24% Trichloroacetic acid (TCA)

100% Trichloroacetic acid (Merck*)	12 ml
Top up to 50 ml with Milli-Q water.	

1 M Tris

Tris base (JT Baker*)	121.12 g
Top up to 1 litre with Milli-Q water	

1X Tris-Buffered Saline (TBS)

1 M Tris	10 ml
5 M NaCl	30 ml
Milli-Q water	700 ml
Adjust to pH 7.5	
Top up to 1 litre with Milli-Q water	

1 M Tris-HCl pH 6.8

Tris base (JT Baker*)	12.112 g
Milli-Q water	50 ml
Adjust to pH 6.8	
Top up to 100 ml with Milli-Q water	

5X Tris Glycine Running Buffer

Tris Base (JT Baker*)	15.1 g
Glycine (Sigma*)	94 g
Milli-Q water	900 ml
10% SDS (BioRad*)	50 ml
Adjust to pH to 8.3	
Top up to 1 litre with Milli-Q water	

15% Tris Glycine Resolving Gel

Milli-Q water	7.2 ml
40% Acrylamide/Bis Solution (BioRad*)	7.5 ml
1.5 mM Tris-HCl pH 8.8	5.0 ml
10% SDS	0.200 ml
10% APS	0.100 ml
N,N,N`,N`- tetramethylethylenediamine (TEMED) (BioRad*)	0.010 ml
Mix well and dispense immediately into plate casting frame.	

4% Tris Glycine Stacking Gel

Milli-Q water	6.36 ml
40% Acrylamide/Bis Solution (BioRad*)	1.0 ml
0.5 mM Tris-HCl pH 6.8	2.5 ml
10% SDS	0.100 ml
10% APS	0.050 ml

N,N,N`,N`- tetramethylethylenediamine (TEMED) (BioRad*)	0.010 ml
Mix well and dispense onto the solidified resolving gel	

0.5 mM Tris-HCl pH 6.8

1 M Tris	0.25 ml
Milli-Q water	200 ml
Adjust to pH 6.8	
Top up to 500 ml with Milli-Q water	

1.5 mM Tris-HCl pH 8.8

1 M Tris	1.5 ml
Milli-Q water	700 ml
Adjust to pH 8.8	
Top up to 1 litre with Milli-Q water and store at room temperature	

Wash/Binding Buffer

$NaH_2PO_4.H_2O$ (Sigma*)	13.8 g
5 M NaCl	30 ml
Milli-Q water	700 ml
Adjust to pH 8.3	
Top up to 1 litre with Milli-Q water	

Western blot transfer buffer

Tris base (JT Baker*)	6.056 g
Glycine (Sigma*)	28.83 g
Milli-Q water	1500 ml
Adjust to pH 8.3-8.6	
Methanol anhydrous (Mallinckrodt*)	400 ml
Top up with Milli-Q water to 2 liter	

X-Gal (40 mg / ml)

X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Fermentas*)	0.4 g
N, N-Dimethylformamide (Sigma*)	10 ml
Protect from light, mix well, aliquot and store in -20°C	

10X YNB (13.4% Yeast Nitrogen Base with Ammonium Sulfate without amino acids) (per liter)

Yeast Nitrogen Base (with ammonium sulfate, without amino acids) (Becton*)	13.4 % (w/v)
*add Milli-Q water and filter sterilize.	

Yeast Extract Peptone Dextrose (YPD) Medium (per Liter)

Yeast extract (Becton*)	10 g
Peptone (Becton*)	20 g
Mili-Q water	900 ml
Autoclave and add to 100 ml of sterile 10x dextrose (glucose).	

Yeast Extract Peptone Dextrose (YPD) Plates (per liter)

Yeast extract (Becton*)	10 g
Peptone (Becton*)	20 g
Milli-Q water	900 ml
Agar (Becton*)	20 g

Autoclave, add to 100 ml of sterile 10x dextrose (glucose), and pour to plates.

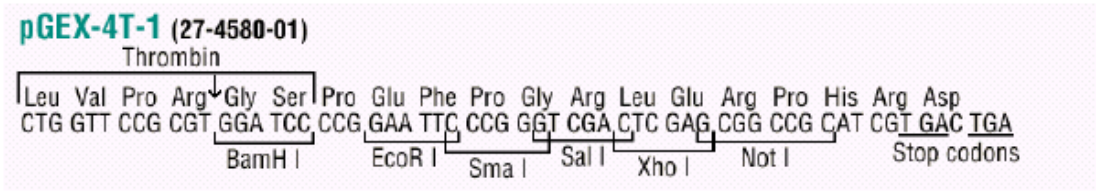
***Note**

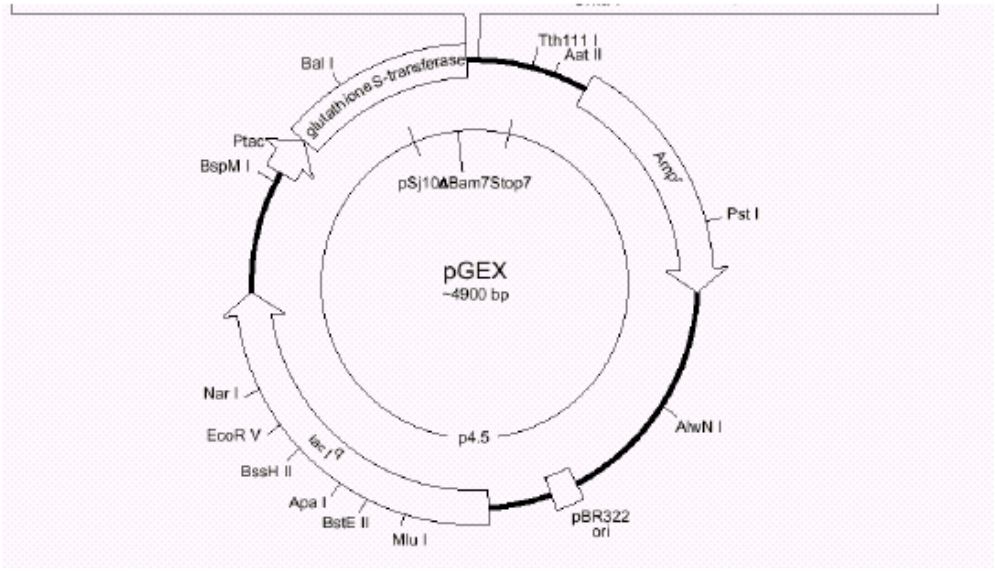
Amresco	Amresco, Solon, Ohio, USA
Becton	Becton Dickinson and Company, Franklin Lakes, NJ, USA
BDH	BDH Laboratory Supplies, Poole, England
BioRad	BioRad, Hercules, CA, USA
BioWhittaker	BioWhittaker Molecular Applications, Rockland, ME, USA
Calbiochem®	Calbiochem®, EMD Biosciences Inc, San Diego, CA, USA
Duchefa	Duchefa, Haarlem, Netherlands
Fermentas	Fermentas, Vilnius, Lithuania
Gibco	Gibco™, Invitrogen Corp, Carlbad, CA, USA
Hayman	Hayman, Essex, England
HyClone	HyClone, Logan, Utah, USA
Janssen	Janssen Pharmaceutica, Beerse, Belgium
JT Baker	JT Baker, Phillipsburg, NJ, USA
Mallinckrodt	Mallinckrodt Inc, Hazelwood, MO, USA
Merck	Merck, Darmstadt, Germany
Roche	Roche Pharma (Schweiz) AG, Basel Switzerland
Sigma	Sigma Chemical Company, St. Louis, MO, USA
Sino	Sino Chemical Co (PTE) Ltd, Singapore
Whitehall	Whitehall Lab Pty Ltd., Punchbowl, Australia

pH are adjusted using either HCl (Merck*) or NaOH (Merck*) unless otherwise specified.
All reagents are stored at room temperature (22-25°C) unless otherwise specified.

Appendix B: Vectors

pGEX-4T-1 expression vector (Amersham Biosciences, Buckinghamshire, England)



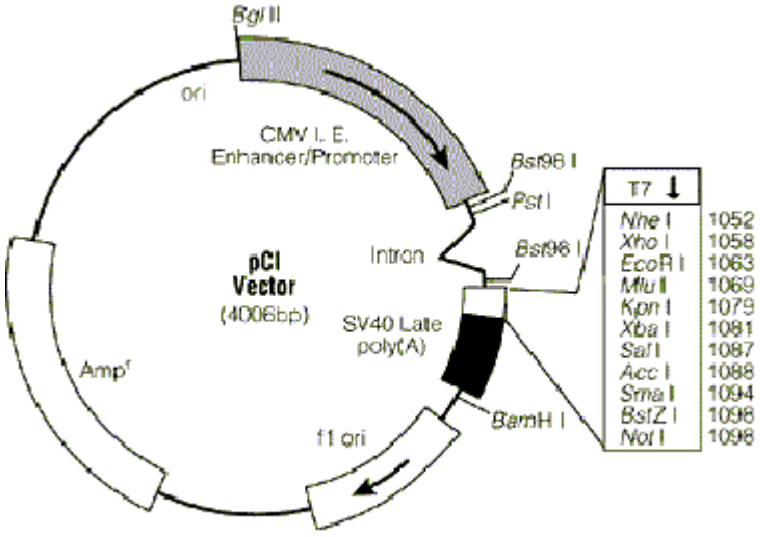


(Source: www.amershambiosciences.com)

pCI mammalian expression vector (Promega, Madison, USA)

Vector size: 4 kb

	Position
Cytomegalovirus immediate-early enhancer/promoter region	1-795
Chimeric intron	857-989
T7 RNA Polymerase Promoter (-17 to +2)	1034-1052
T7 promoter transcription start site	1051
Multiple cloning region	1052-1104
SV40 late polyadenylation signal	1111-1332
Phage f1 region	1422-1877
Beta-lactamase (AmpR) coding region	2314-3174



pCI mammalian expression vector map
(Source: www.Promega.com)

Der p 5 leader sequence

BspE1

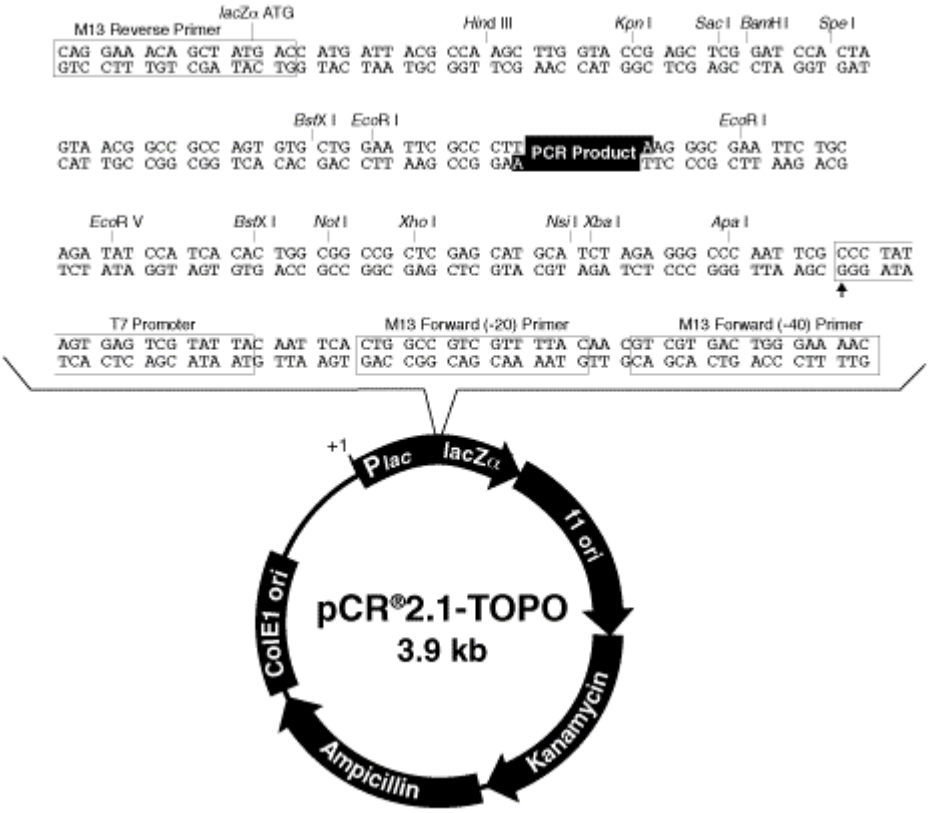
```
5′ TCGAGATCAATCATGAAATTCATCATTGCTTTCTTTGTTGCCACTTTGGCAGTTATGACTGTTTCCGGA 3′
3′ AGCTCTAGTTAGTACTTTAAGTAGTAACGAAAGAAACAACGGTGAAACCGTCAATACTGACAAAGGCCT 5′
```

Sequences in bold is the Der p 5 sequence. Underlined sequence is the restriction enzyme site of BspE1.

pCR®2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA)

Vector size: 3.9kb

	Position
LacZα fragment	1-571
M13 reverse priming site	205-221
Multiple cloning site	234-357
T7 promoter / priming site	364-383
M13 Forward (-20) priming site	391-406
M13 Forward (-40) priming site	411-426
F1 origin	548-962
Kanamycin resistance gene	1296-2090
Ampicillin resistance gene	2108-2968
ColE1 origin	3113-3786



pCR®2.1-TOPO vector
(Source: www.Invitrogen.com)