

Allergenicity and Cross-Reactivity of 3 House Dust Mite Species Among Filipino Allergic Patients

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The immunoglobulin E-binding activity of allergens from the house dust mite (HDM) *Blomia tropicalis* (Bt), *Dermatophagoides farinae* (Df), and *Dermatophagoides pteronyssinus* (Dp) were determined using a panel of 210 allergic and 85 non-atopic Filipino subjects. Enzyme-linked immunosorbent assay showed that 91%, 89%, and 86% of Filipino allergic subjects tested are sensitized with Bt, Df, and Dp allergens, respectively. Western Blot Analysis identified multiple IgE reactivities of selected patients' sera to HDM proteins with molecular weight ranging from 10–80 kDa. Absorption assays by inhibition ELISA showed that up to 60% inhibition can be detected between the three HDM species. The results obtained in this study suggest that Bt, Dp, and Df are important HDM species in the local population causing sensitization in the majority of allergic Filipino patients tested. The incorporation of Bt, Dp, and Df allergens in the panel of diagnostic allergens for HDM allergy are highly recommended.

Key Words: Allergy, Immunoglobulin E, *Blomia tropicalis*, *Dermatophagoides farinae*,
Dermatophagoides pteronyssinus

INTRODUCTION

Allergy is a hypersensitivity reaction initiated by immunological mechanisms characterized by the production of elevated levels of allergen-specific immunoglobulin E (IgE). In atopic individuals, the immune system reacts inappropriately to allergens, resulting in the development of inflammatory allergic diseases such as allergic asthma (wheezing, coughing, shortness of breath, and chest tightness), rhinoconjunctivitis (congestion, sneezing, itching, and nasal discharge), and atopic dermatitis or eczema (itching, rashes of the skin, and lesions). The worldwide incidence of allergic diseases is reaching epidemic proportions (Holgate 1999). Epidemiological studies show that 10-30 % of the world population is afflicted with allergic diseases (The ISAAC Steering

Committee 1998) and the costs to public health and the economy are substantial and growing (Cookson 1999). Allergic diseases are major contributors to morbidity and mortality of the world population. Most often, allergies are caused by the immune reaction to common inhaled proteins called aero-allergens. The most frequently implicated indoor allergen sources are the house dust mites (HDM). HDM have been well recognized to play an important role in the pathogenesis of allergic diseases (Platts-Mills and Chapman 1987).

HDM allergens sensitize and induce allergies in a large portion of atopic individuals worldwide, but there is a dearth of information on the sensitization profiles of allergic Filipinos to allergens found in HDM. The most common HDM species include *Dermatophagoides pteronyssinus* (Dp), *Dermatophagoides farinae* (Df), and *Blomia tropicalis* (Bt). Sensitization to HDM is

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the major independent risk factor for asthma as shown by epidemiological studies in Singapore (Chew et al. 1999); Japan (Ohshima et al. 2002); Hong Kong (Leung et al. 2002); China (Wong et al. 2002); and in many other countries. It has been demonstrated that a dose response relationship exists between the level of exposure to HDM allergens and the severity of asthma, with the risk of HDM-sensitized children having asthma doubling for every doubling level of major allergens (Peat et al. 1996). Approximately 90-96% of atopic populations Singapore and Taiwan are sensitized to HDM allergens (Kuo et al. 1999). Nasal provocation studies with persistent allergic rhinitis (PAR) patients showed that HDMs such as *B. tropicalis* extract could induce asthmatic symptoms (Wang et al. 2003). Aqueous extracts prepared from cultures of these HDMs can elicit skin-test responses and radioallergosorbent-test activity, thus are invaluable diagnostic reagents for HDM allergy (Arlian and Platts-Mills 2001). Furthermore, HDM extracts are used in immunotherapeutic procedures. Understanding the sensitization profiles of common HDM species in the local population of allergic patients will establish a basal pool of information that could help allergologists design highly specific and accurate diagnostic and therapeutic strategies for HDM allergy among Filipinos.

MATERIALS AND METHODS

Study Subjects

The study included 210 doctor-diagnosed Filipino atopic subjects with allergic asthma, atopic dermatitis and/or allergic rhinitis seen at the University of Santo Tomas Hospital's Allergy and Clinical Immunology and Otorhinolaryngology Sections of the Out-Patient Department and Dermatology clinics (Table 1); and

85 healthy, non-atopic Filipino subjects. Subjects were asked to fill out a Patient Information Sheet that asked for basic information and their medical history with allergy. The patients' phenotypes considered were based on the answers to questions from international standardized questionnaires of the International Study of Asthma and Allergy in Childhood (ISAAC) and the International Primary Care Airways Group (IPAG) core questions. Control subjects include individuals without history of allergy and without immediate relatives with allergy.

Collection of Blood Samples

An average of 5 mL whole blood sample was collected from each subject using a sterile 5 mL Terumo syringe with a gauge 21 needle. Blood samples were allowed to stand for 1 h at room temperature. Serum samples were isolated by centrifugation and transferred into tubes in aliquots and stored at -20° C until use.

HDM Aqueous Extract Preparations

Five grams (wet weight) of frozen Bt, Dp or Df (a kind gift from Prof. Chua Kaw Yan, National University of Singapore) was wrapped in aluminum foil and soaked in liquid Nitrogen for 5 min. The frozen mites were mechanically ground using a precooled mortar and pestle for 30 min. Extraction was performed using a total volume of 20 mL of 1X Tris-Buffered Saline (TBS) with 2 mM Phenyl Methyl Sulfonyl Fluoride, PMSF, (Sigma-Aldrich, Saint Louis, MO, USA), and 1 mM EDTA (BioRad, Hercules, CA, USA) added slowly while grinding the mites. The mite extract suspension was incubated for 16 h at 4° C with constant shaking. After centrifugation at 17,000 rpm for 20 min using the Sorvall[®] Ultra Pro 80 centrifuge (Kendro Lab. Products, Newtown, CT, USA), the supernatant was collected and quantitated by BioRad DC Protein Assay. Aqueous Bt protein extracts were stored in aliquots at -80° C until use.

SDS-PAGE

Aqueous HDM extracts were analyzed by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). Protein samples were mixed 1:1 with 2X SDS-PAGE sample buffer and were boiled for 10 min. Samples were separated on a 15% Tris-Glycine gel using the Mini PROTEAN electrophoresis system (BioRad, Hercules, CA, USA). Gel was run at 110 Volts for 90 min. Broad Range Marker (BioRad, Hercules, CA, USA) was used as standard. SDS-PAGE gel was stained with Coomassie brilliant blue R-250 (BDH laboratory Supplies, Poole, England).

Table 1. Diagnosed subjects with allergic asthma, atopic dermatitis and/or allergic rhinitis

		Number	Percentage (%)
Age	0-20	154	73.33
	21-50	56	26.66
Sex	Male	101	48.09
	Female	109	51.91
Type of Allergy	Allergic Asthma Only	67	31.91
	Allergic Rhinitis Only	62	29.52
	Atopic Dermatitis Only	43	20.47
	Combination of the Above	38	18.10
Residence	Metro Manila	142	67.62
	Outside Metro Manila*	68	32.38

*Bulacan, Pampanga, Quezon, Laguna, Cavite and Batangas

Enzyme-Linked Immunosorbent Assay

Enzyme linked immunosorbent assay (ELISA) was used to evaluate the profile of sensitization of a panel of Filipino allergic patients' and non-atopic control sera to the HDM extracts following the methods described by Ramos et al. (2003). Briefly, 100 µg/mL of the HDM aqueous extracts were prepared and coated onto ELISA plates overnight at 4° C using 50 µL of 0.1 M NaHCO₃, pH 8.3. Plates were blocked with 1% BSA (Sigma) in Phosphate Buffered Saline with 0.05% Tween 20 (PBS-T) for 1 h at room temperature. ELISA plates were incubated overnight with 5x diluted human sera, then for 1 h at room temperature with biotinylated anti-human IgE (Pharmingen, CA, USA) diluted 1000x in blocking buffer. Plates were incubated with 2000x dilution of ExtrAvidin-alkaline phosphatase conjugate (Sigma) for 1 h. Finally, colorimetric reaction was performed using p-nitrophenyl phosphate (Sigma). Absorbance at 405 nm was determined using an ELISA reader. Human IgE (Pharmingen) was used as a standard per plate in the calculation of IgE concentration. The mean + 2 standard deviations of the Bt, Dp and Df allergen-specific IgE levels of the 85 non-allergic control subjects were used as cut-off values in determining a positive reaction among the allergic patients used.

Western Blot Analysis

The IgE reactivity of selected sera to specific allergens found in the three HDM extracts was determined by WBA as previously described (Ramos et al. 2003). In brief, Bt, Dp, and Df aqueous extracts (0.5 µg) were electrophoresed on a 15% Tris-Glycine gel and electroblotted onto Hybond-C Nitrocellulose membrane (Amersham Life Sciences, Buckinghamshire, England) using the MiniProtein 3 cell (BioRad, Hercules, CA, USA) at 110 V for 1 h. The membrane was blocked with 5% skimmed milk in PBS-T. After overnight incubation with selected human sera (5x dilution) in blocking buffer at 4° C, the membrane was incubated with biotinylated anti-human IgE (Sigma-Aldrich, Saint Louis, MO, USA). Finally, the membrane was incubated with peroxidase-conjugated ExtrAvidin (Sigma-Aldrich, Saint Louis, MO, USA). The membrane was washed 6x with PBS-T between steps. Results were detected by incubation with Alkaline Phosphatase Color Development Solution (BioRad).

Inhibition Assay

Absorption assay by ELISA was performed to determine the cross-reactivity of the allergens found in the three HDM extract preparations. A 96-well ELISA plate (Corning, NY, USA) was coated with HDM extracts (10 µg/mL) as described above. Seven selected human serum samples were pre-absorbed (14 h) separately with HDM extracts to a final concentration of 10 µg/µL. ELISA was

performed as described above. Percentage inhibition was computed using the following formula: % inhibition = $[(A_{\text{unabsorbed}} - A_{\text{absorbed}}) / A_{\text{unabsorbed}}] * 100$; where $A_{\text{unabsorbed}}$ is the OD₄₀₅ of serum samples not pre-absorbed with antigen and A_{absorbed} is the OD₄₀₅ of serum samples pre-absorbed with an HDM extract.

RESULTS AND DISCUSSION

Aqueous extracts of Bt, Dp, and Df were prepared by mechanical and chemical digestion in the presence of protease inhibitors. Bio-Rad DC Protein Assay showed that 158.76 mg, 174.24 mg, and 186.45 mg of total HDM proteins were isolated from 500 mg whole body (wet weight) of Bt, Dp, and Df, respectively. The 3 HDM extract preparations registered different SDS-PAGE protein profiles as shown in Figure 1. Under a 15% Tris-Glycine Gel, protein bands observed from the HDM

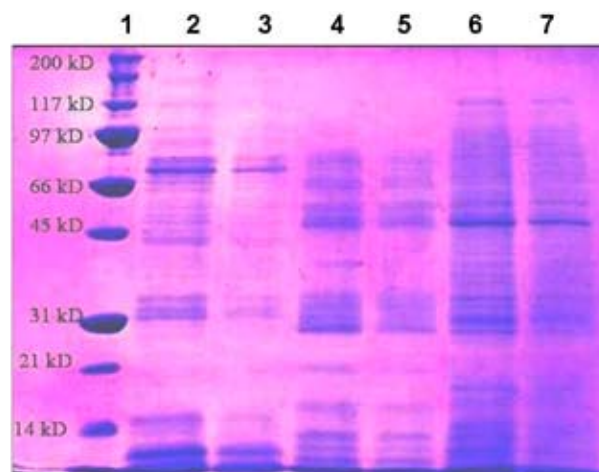


Figure 1. Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE) profile of Bt (lanes 2 and 3), Dp (lanes 4 and 5) and Df (lanes 6 and 7) aqueous extract preparations. Aqueous extracts at 150 µg (lanes 2, 4 and 6) and 100 µg (lanes 3, 5, and 7) together with protein marker (lane 1) were electrophoresed on a 15% Tris-Glycine gel using a BioRad Mini-PROTEAN system.

aqueous extracts ranged from ~10-150 kDa. Bt extract contains three major protein bands having molecular weights of approximately 70, 33, and 10 kDa. On the other hand, Dp and Df aqueous extracts registered nearly similar protein banding patterns with observed major bands between 66 and 45 kDa, and bands between 31 and 21 kDa. The protein banding patterns of the three HDM aqueous extracts obtained in this study are comparable with SDS-PAGE results previously reported (Yi et al. 1999). The presence of intact protein bands ranging from

low to high molecular weight proteins indicates the good quality of the allergen extracts used in the succeeding allergenicity assays performed in this study.

HDMs are distributed worldwide but their prevalence varies from one region to another (Arlian & Platts-Mills 2001). Domestic mites belonging to genus *Dermatophagoides* and *Euroglyphus* account for 90 percent of the mite species in house dust from temperate regions (Platts-Mills et al. 1997). Dp is the dominant HDM species in constantly damp climates. On the other hand, Df survives better in drier climates than Dp, and it is the most predominant mite species in areas with prolonged dry winters. Bt is a domestic mite of clinical importance in tropical and subtropical regions. A survey of the acarofauna in selected houses of allergic patients in Metro Manila revealed that *Dermatophagoides* and *Blomia* species are the most abundant HDM species (Ramos et al. 2006). In addition, four HDM species not previously reported in Philippine dust samples were also identified including *Chortoglyphus arcuatus*, *Euroglyphus* sp, *Haplochthonius simplex*, and *Tydeus* sp. In the same study, an average mite density of 206 mites/gram of dust, an HDM count within the range considered as risk factor for allergy sensitization, was also reported (Ramos et al. 2006). HDMs feed on human and animal scales colonized by microfungi, yeasts, and bacteria. Mites can be found in floors and tend to bury themselves deep in carpets, mattresses, and soft furnishings.

HDMs are important sources of allergens that trigger allergic sensitizations among atopic individuals. Thus far, there are nineteen groups of identified and characterized HDM allergens belonging to ten HDM species (Thomas et al. 2002; Kawamoto et al. 2002). Of the nineteen denominated HDM allergens, 7 groups (groups 1, 2, 3, 9, 11, 14, and 15) are considered major allergens based on the reported frequency of IgE binding. Although most of the known HDM allergens are catalytic proteins (groups 1, 3, 4, 6, 8, 9, 15, and 18); at least two are structural proteins (groups 10 and 11) and a few with special or unknown functions. Most of the known HDM allergens are low-molecular weight proteins, but a few are high-molecular-weight allergens with a molecular weight above 60,000 Daltons (Thomas et al. 2002; Kawamoto et al. 2002).

Results of IgE enzyme-linked immunosorbent assay (Figure 2) showed 91% (192/210), 89% (187/210), and 86% (180/210) positive reaction with Bt, Df, and Dp aqueous extracts, respectively. Unpaired t-tests showed a significant difference between the Bt ($p=0.0054$), Df ($p=0.0019$) and Dp ($p=0.0026$) allergen-specific IgE levels of allergic patients with the Bt, Df and Dp allergen-specific IgE levels of non-atopic subjects tested. Bt and

Dermatophagoides sp. have been regarded as important causative agents of mite-related allergic diseases worldwide (Arlian et al. 2002). It has been previously reported that Bt is responsible for sensitization of 70-90% of asthmatic sera in tropical Singapore and Malaysia (Chew et al. 2001) while Dp and Df are clinically significant in temperate regions (Ulrik and Backer 2000). A study reported by Cua-Lim (1990) in 1990 showed that selected asthmatic patients' sera from Metro Manila are highly reactive to allergens from Dp and Df extracts. Interestingly, results obtained in this study showed that other than Dp and Df, allergens from Bt trigger allergic sensitizations in the majority of Filipino allergic patients' tested. This study confirmed that *Blomia tropicalis* is a clinically important house dust mite species in the Philippines.

Detailed analysis of the profile of IgE reactivity of the 210 allergic subjects showed that 95% (200/210) are sensitized with allergen from any of the three HDMs tested. Furthermore, 80% (169/210) of the subjects tested showed triple positive IgE-binding reactions to Bt, Dp, and Df allergens. These results indicated the high prevalence of HDM sensitization among Filipino allergic patients. Further analyses of the IgE reactivities of the subjects tested showed that 2% (5/210) and 1% (3/210) reacted positively to Bt and Df allergens only, respectively, while none reacted to Dp allergens only. Double positive reactions to Bt and Dp; Bt and Df; and Dp and Df were computed at 3% (7/210); 5% (11/210); and 2% (4/210), respectively. Presence of cross-reactive allergens from different HDM species might be responsible for the observed multiple IgE reactivities.

Protein bands observed in the SDS-PAGE that binds IgE from allergic patients' sera were examined by WBA. Ten sera that registered positive reactions to the three HDM species as determined in the ELISA were selected for IgE-binding activity by WBA. Multiple reactivities of serum IgE to proteins with molecular weight ranging from 20–80 kDa were observed in the HDM aqueous extract preparations. Majority of the patients' IgE reacted to an approximately 40 kDa protein in the Bt extract (indicated by an arrow in Figure 3A). On the other hand, most of the patients' IgE, are reactive to an approximately 80 kDa protein (allergen) in the Dp extract (indicated by an arrow in Figure 3B). The presence of minor IgE-reactive protein bands of <20 kDa and approximately 30 kDa, 50 kDa, and 60 kDa were also noted. These IgE-reactive proteins contribute to the allergic sensitization among local allergic patients tested. In a related study, about 25 IgE-binding components between 11 and 85 kDa have been shown (Caraballo et al. 1993). The most frequently detected allergens with IgE-binding reactivity are the 11–14 kDa, 33 kDa, 36 kDa and 64 kDa allergens. The presence of

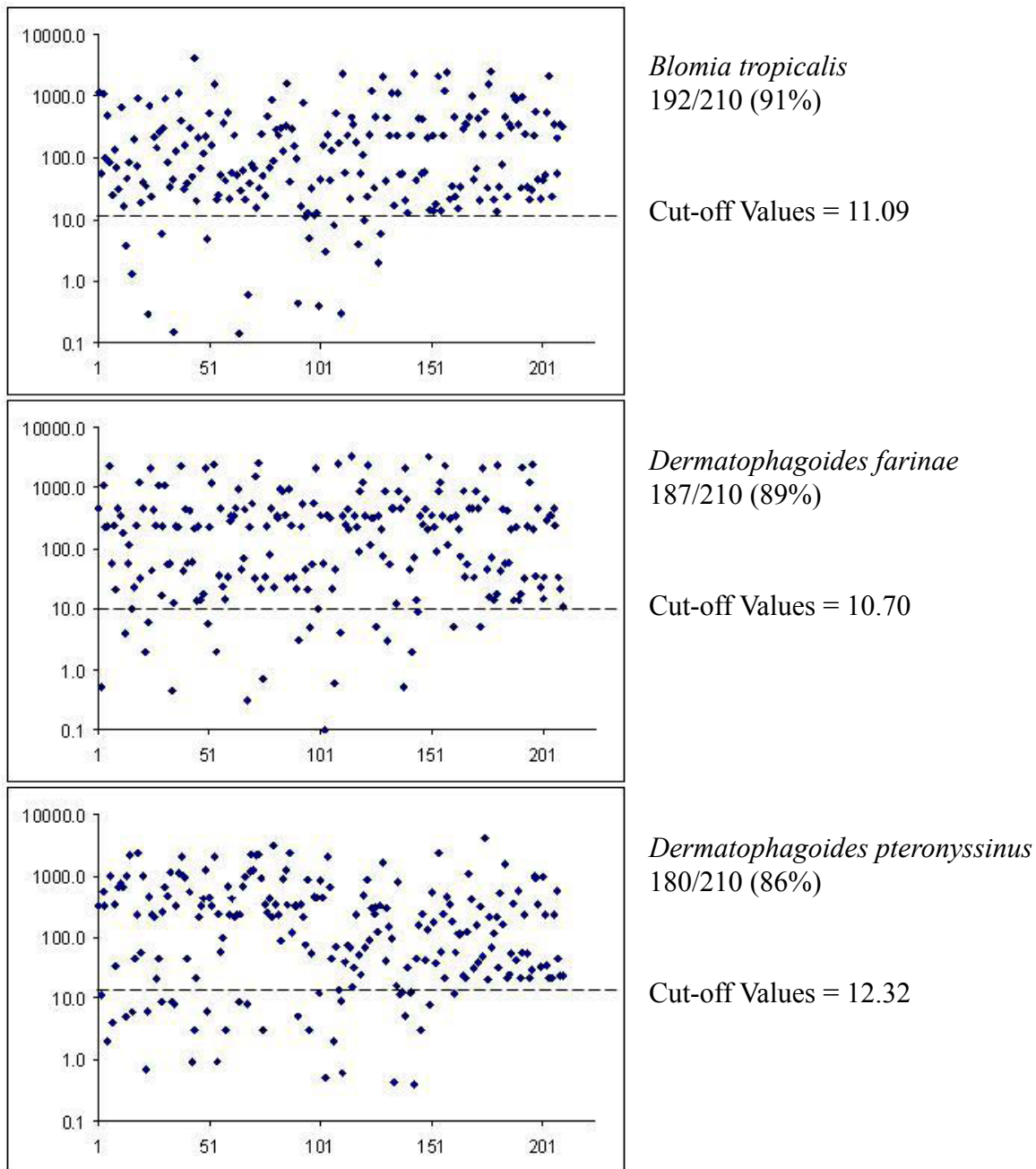


Figure 2. Summary of the profile of sensitization of 210 Filipino allergic patients against Bt, Df and Dp extracts by IgE ELISA. The cut-off value represented by a horizontal bar was computed as mean + 2 standard deviation of the 85 non-allergic control subjects

low molecular weight allergens including the group 2 and 5 allergens, however, cannot be ascertained in the present study as the gels used in the Western blot analysis do not show molecular weights below 20 kDa.

Cross-reactivity between the allergens found in the three HDM species was also shown in this study (Figure 4). Df and Dp absorbed sera incubated with Bt extracts have an average percentage of inhibition of 52% and 53%,

respectively. On the other hand, Bt-absorbed and Df-absorbed sera incubated to Dp extracts showed 15% and 82% inhibition, respectively. The sera that were absorbed with Bt and Dp incubated to Df extracts gave 29% and 97% inhibition, respectively. The results showed that cross-reactive allergens are present among the three HDM species and the degree of cross-reactivity varies between patients. The observed variation in the inhibition capacity of the three HDM allergens is reflected by the computed

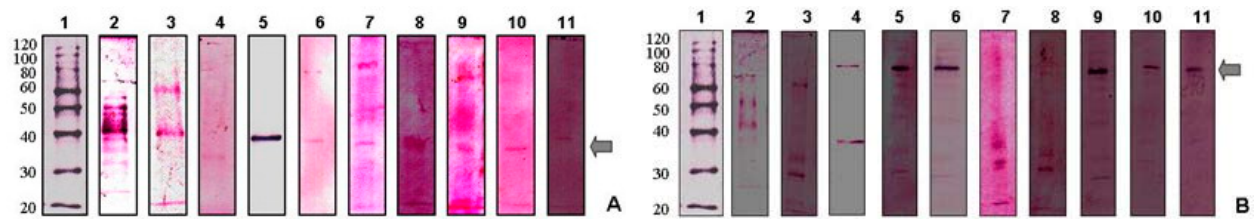


Figure 3. Western blot analysis showing the IgE reactivity of major protein bands from Bt (A) and Dp (B) aqueous extracts using ten selected allergic patients' sera. Lane 1 is a protein marker (Invitrogen Magic Marker Western Standard) while lanes 2-11 represents different sera from selected allergic patients

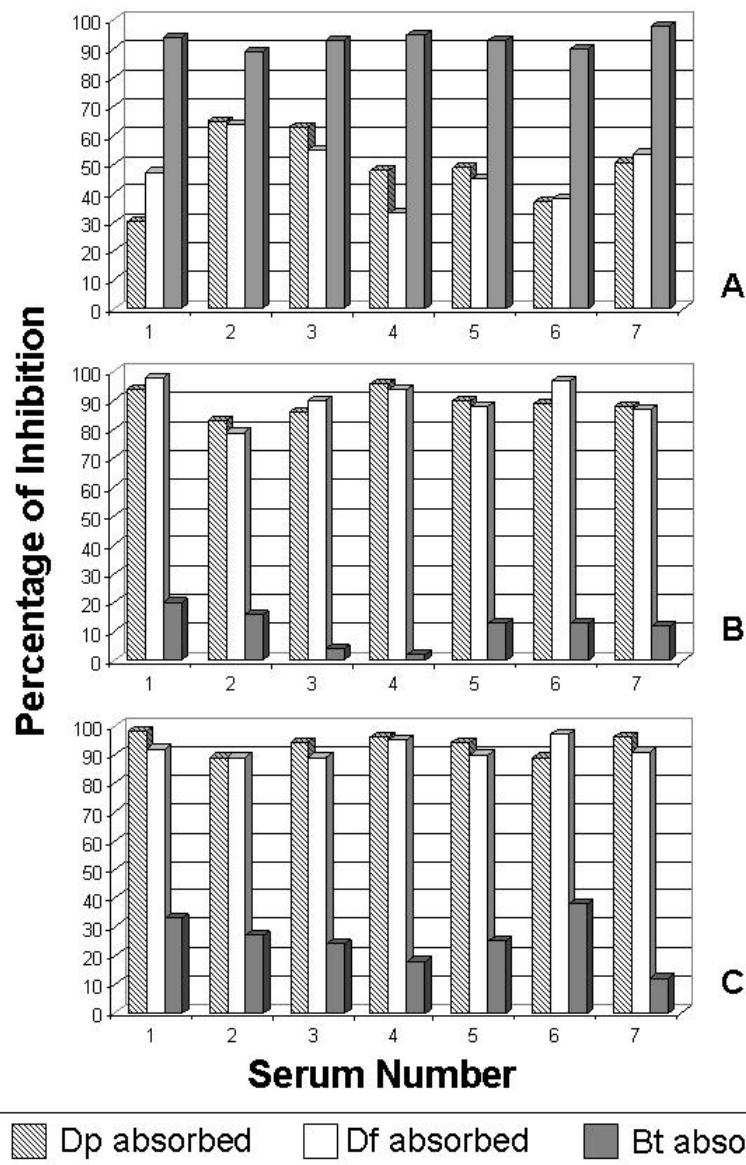


Figure 4. IgE Inhibition ELISA with seven sera against Bt (A), Df (B) and Dp (C) aqueous extracts. Each serum were separately pre-absorbed with Dp, Df or Bt allergen extracts prior to ELISA in plates coated with Bt (A), Df (B) and Dp (C) aqueous extracts. Percentage inhibition refers to the difference in the OD405 values between unabsorbed and absorbed sera divided by the former and expressed as a percentage

standard deviation of the percentage of inhibition where Dp extract absorption exhibited 12.6%, 4.5%, and 3.5% when reacted to Bt, Dp and Df extracts, respectively. Likewise, the percentage of inhibition of Df extract deviates by 10.6%, 6.6%, and 3.0% when reacted with Bt, Dp, and Df extracts, respectively. Deviation in the percentage of inhibition was also observed in Bt at 3.0%, 6.4%, and 8.7% when reacted with Bt, Dp, and Df extracts, respectively (Figure 4). Majority of the allergens from the *Dermatophagoides* spp are cross-reactive to allergens from other species, while most of the allergens from Bt are species-specific. In previous studies, immunoabsorption experiment showed that most of the IgE antibodies to Bt (64%) reacted with species-specific allergens, while 36% are cross-reactive to Dp (Arruda et al. 1991). Cross-reactivity between Dp and Df was also reported previously (Ferrandiz et al. 1995).

Studies have shown that cross-reactivity between allergens from different house dust mite species exists. IgE antibodies from mite-allergic patients have been found to cross-react with different house dust mite species but some IgE antibodies exhibit specificities unique to a particular HDM species (Griffin et al. 1989). These observed cross-reactivities between the three HDM species may indicate complexity in the profile of allergens that cause allergic sensitization among atopic individuals. Furthermore, different house dust mite species usually co-inhabit homes and allergens found on these dust mites may cause parallel allergic sensitization to susceptible individuals.

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SUMMARY AND CONCLUSION

The results obtained in this study suggest that *Blomia tropicalis*, *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* are important house dust mite species in the local population causing sensitization in the majority of allergic Filipino patients tested. The incorporation of Bt, Dp and Df allergens in the panel of diagnostic and immunotherapeutic allergens for HDM allergy are highly recommended.

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