ORIGINAL ARTICLE

Skin Prick Tests and Enzyme-Linked Immunosorbent Assays among Allergic Patients Using Allergenic Local Pollen Extracts

Mary Anne R. Castor, MD,[1*] Maria Katrina Diana M. Cruz, RCh, MSc,[2*] Krystal M. Hate, MSc,[2] Gregg Austine M. Balanag, BS,[2] Roche Dana C. Reyes, MD,[1] Maria Socorro Agcaoili-De Jesus, MD,[3] Cherie C. Ocampo-Cervantes, MD[3] and Leslie Michelle M. Dalmacio, PhD[2]

[1]*Division of Allergy and Immunology, Department of Pediatrics, Philippine General Hospital, University of the Philippines Manila*
[2]*Department of Biochemistry and Molecular Biology, College of Medicine, University of the Philippines Manila*
[3]*Division of Allergy and Immunology, Department of Medicine, Philippine General Hospital, University of the Philippines Manila*

ABSTRACT

Background. Allergic respiratory diseases are prevalent in the Philippines, with allergic rhinitis and asthma occurring at 20% and 8.7% of the population, respectively. The diagnosis of respiratory allergies is achieved by a combination of patient history and different screening tools, especially for the identification of the allergic triggers such as allergy skin prick test (SPT) and serum-specific IgE enzyme-linked immunosorbent assays (sIgE ELISA). The Philippines, being a tropical country, have a wide variety of plant species with potential to produce allergenic pollen grains. Knowledge of the sensitization profiles of Filipino allergic patients to our local pollen allergens is currently limited.

Objectives. The aim of this study is to determine the sensitization profile of patients with respiratory allergies (allergic rhinitis and/or asthma) through the allergy skin prick test (SPT) using allergenic local pollen extracts. It also aimed to determine if there is a positive agreement between the SPT and sIgE ELISA positivity rate and whether the results have relationship with the pollen purity and the protein content of the extracts.

Methods. Pollen allergens were extracted from *Amaranthus spinosus* (pigweed), *Mimosa pudica* (makahiya), *Tridax procumbens* (wild daisy), *Imperata cylindrica* (cogon), *Oryza sativa* (rice), *Pennisetum polystachion* (foxtail grass), *Sorghum halepense* (Johnson grass), *Albizia saman* (acacia), *Cocos nucifera* (coconut), *Leucaena leucocephala* (ipil-ipil), and *Mangifera indica* (mango). SPT was performed at the Allergy Clinic of the University of the Philippines-Philippine General Hospital on patients with allergic rhinitis and/or bronchial asthma. Blood samples were collected from patients who developed wheal diameters of 3 mm or more than the negative control. Sera were tested against the same pollen extracts using ELISA.

Results. Of the one hundred sixty-five (165) patients who submitted for skin prick test, 129 showed positive SPT results to the pollen extracts. Weeds were the most sensitizing (51.9%-58.1%). Blood samples were collected from these patients and tested for sIgE ELISA and among them, 71 were positive in the sIgE ELISA. Highest sensitization rates in sIgE ELISA were found in coconut, pigweed, Johnson grass, and rice. The highest positive agreements or the proportion of patients with positive sIgE ELISA among those with positive SPT were in coconut, followed by Johnson grass, pigweed, and rice. Most of the pollen sensitized patients on SPT are polysensitized.

**Dr. Castor and Ms. Cruz share first authorship for this manuscript.*

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Corresponding author: Maria Katrina Diana M. Cruz, RCh, MSc
Department of Biochemistry and Molecular Biology
College of Medicine
University of the Philippines Manila
547 Pedro Gil St., Ermita, Manila 1000, Philippines
Email: mmcruz8@up.edu.ph
ORCiD: https://orcid.org/0009-0006-9064-6172

Conclusion. SPT is a safe, simple, and rapid method for the diagnosis of IgE-mediated allergy. The lower number of positive patients in sIgE ELISA may be attributed to the low serum IgE levels and low quantities of effectual allergen components in extracts. Results of both SPT and ELISA must be correlated with a patient's clinical history, particularly the patient's exposures, and physical examination.

Keywords: pollen, rhinitis, allergic, asthma

INTRODUCTION

Wind-driven pollen plays an important role in plant ferti-lization; wherein seeds are formed once the male gametes, or sperm, contained in the pollen comes in contact with the female gametes (ovules). It is also a common trigger of allergic diseases such as allergic rhinitis and asthma. Although allergic reaction to house dust mites among patients at the Otolaryngology-Allergy clinic of St. Luke's Medical Center between 2011-2016 is reported to be 97.4%, grass is the most common outdoor allergen in the country with 67% and 58.7% of patients being skin-prick test positive to Bermuda and Johnson grasses, respectively.[1] In a study by Estrella et al.[2] that included 289 patients (62% male, mean age 9.03 years), most of the patients were allergic to house dust mite (74%); whereas among the outdoor allergens, Johnson grass (10%), pigweed (5.2%), and mango tree pollen (5.2%) were the most common. The Philippines, being a tropical country, have a wide variety of plant species with potential to produce allergenic pollen grains. In a study by Sabit et al.[3], the most predominant pollen types in Manila during November 2013 to October 2014 were Urticaceae, Cannabaceae, and Poaceae at 38%, 16%, and 10% prevalence, respectively, and most of the total airborne pollen were obtained during the dry season. Excess carbon dioxide in the atmosphere causing global temperature to rise, as well as improvement in photosynthesis and reproductive effects, resulted to increase in pollen production[4], and to increase in pollen potency or allergen content[5]. Thus, increase in allergic respiratory diseases due to climate change is an alarming concern of late. Allergic respiratory diseases, especially allergic rhinitis, may not be life-threatening diseases but they affect the quality of life of the patients. Hence, early diagnosis and control of symptoms are crucial.

The diagnosis of respiratory allergies is achieved by a combination of patient history and different screening tools, especially for the identification of the allergic triggers. Identification of triggers is important in a disease that can only be controlled. Skin prick test (SPT) is a sensitive and reproducible technique using standardized allergenic extracts. This test identifies allergen-specific immunoglobulin E (sIgE) bound to the high affinity receptor of mast cells (FcΣRI) expressed on the membrane of skin mast cells[6] and gives positive result when at least a wheal diameter of 3-mm more than the negative control develops. The serum-specific IgE test is an in-vitro test which measures specific IgE in the serum of patients. This test is usually done when the patient cannot tolerate SPT or has dermatographism or extensive atopic dermatitis or in patients who cannot be put off antihistamines. Clinical history, SPT, and serum sIgE measured by ImmunoCAP system are the standard work-up for diagnosing allergic rhinitis and/or bronchial asthma and for deciding on allergen immunotherapy prescription in atopic patients.[7] However, sample dependency remains a concern in serum-sIgE tests, especially because some patients have low sIgE levels as only after the saturation of the whole receptor system of the organism can IgE be found free in serum and other biological fluids.[6] Knowledge of the sensitization profiles of Filipino allergic patients to our local pollen allergens is currently limited.

Therefore, the primary objective of this study is to determine the sensitization profile of patients with respiratory allergies (allergic rhinitis and/or asthma) to allergenic local pollen extracts through SPT. Secondary objectives are to determine if there is a positive agreement between the SPT and sIgE ELISA positivity and whether the pollen purity and protein concentration of the extract would have a relationship with the results of the SPT and sIgE ELISA.

MATERIALS AND METHODS

This study is a part of a bigger study wherein inclusion requires a SPT-positive result. Thus, blood samples were collected and sIgE ELISA was performed for the patients who satisfied the inclusion criteria.

Study Design

This is a descriptive study of the SPT and sIgE ELISA of allergic patients using allergenic local pollen extracts.

Study Setting and Subjects

Patients, aged 19-50 years, with allergic rhinitis and/or bronchial asthma, who consulted at the Allergy Clinic of the Philippine General Hospital and whose symptoms were controlled, were recruited from July 2021 to February 2022. Posters inviting volunteers with symptoms of allergic rhinitis and/or asthma were displayed around the Philippine General Hospital and at UP Manila, and through social media. Patients were screened for intake of antihistamines that may suppress the skin test reaction and for symptoms, such as cough, that might lead to an asthma flare.

An informed consent was obtained prior to the procedure. The study procedure was explained to the participants and questions were entertained. The screening, skin test, and blood extraction were done at either the Allergy Clinic or the Pediatrics Clinical Research Unit of the Department of Pediatrics in PGH by a fellow-in-training of the Division of Allergy and Immunology. Patients with one or more positive reactions to the pollen allergen extracts underwent blood

extraction. No blood extraction was done for patients with negative skin tests to the extracts.

Pollen Collection

Pollen samples of the most common allergenic pollen *Amaranthus spinosus* (pigweed), *Mimosa pudica* (makahiya), *Tridax procumbens* (wild daisy), *Imperata cylindrica* (cogon), *Oryza sativa* (rice), *Pennisetum polystachion* (foxtail grass), *Sorghum halepense* (Johnson grass), *Saccharum spontaneum* (talahib), *Albizia saman* (acacia), and *Leucaena leucocephala* (ipil-ipil) were collected from different areas in the country. Anthers were separated from the flowers through gentle brushing directly to the test sieve with mesh sizes of 25 µm, 38 µm, 45 µm, or 150 µm depending on the size of the pollen. *Cocos nucifera* (coconut) pollen grains were provided by the Philippine Coconut Authority Zamboanga Research Center. *Mangifera indica* (mango) whole anthers containing the pollen were plucked using tweezers. Pollen grains were visualized and measured using OPTIKA Microscope PROview Software under LPO (40X) and HPO (400X). Pollen grains were stored at -20 °C prior to extraction. Pollen and debris or contaminants present in a sample were counted in a haemocytometer. Percent pollen purity was calculated using the formula:

$$\% \text{ purity} = \frac{\text{total no. of intact target pollen sample}}{\text{total no. of target pollen and impurities}} \times 100$$

The percent purity of each sample was calculated using the average purity of three trials.

Pollen Extraction

Pollen samples were defatted with 1:5 (w/v) ratio of pollen to diethyl ether at room temperature thrice for 50 minutes each with shaking then air-dried for 24 hours. Allergens were extracted using 1:10 (w/v) ratio of pollen to 1X Phosphate-buffered saline (PBS, pH 7.3) with 0.3% protease inhibitor overnight at 4°C. The extract was centrifuged at 12,000 rpm, 4°C for 30 minutes. Supernatant was serially filtered using 0.80, 0.45, and 0.20 µm Whatman syringe filters and stored at -20°C until further use.

Lowry assay was performed to determine the total protein concentration of each pollen extract. Pollen samples were diluted to a 1:10 (v/v) ratio of pollen samples to 1X PBS. One hundred microliter (100 µL) of the standard or sample solution was mixed with 25 µL of 5X Copper-Tartrate solution in the microplate wells and incubated for 10 minutes at room temperature in the dark. After incubation, 10 µL of 1:1 ratio of Folin-Ciocalteu and 0.1M NaOH was mixed in. Plate was incubated for 20 minutes at room temperature in the dark. Absorbance value was measured at 630 nm using SPECTROstar Nano microplate reader. Bovine Serum Albumin (100-5000 µg/mL) was used as standard for the calibration curve.

Skin-prick Test

Prior to performing the procedure, the patients (n=165) were screened for symptoms and intake of antihistamines and other medications that may suppress the result of the SPT. All patients with an intake of long acting antihistamine starting seven days prior until the day of skin testing were excluded. Patients with uncontrolled allergic rhinitis and/or asthma symptoms were also excluded.

SPT was performed on the volar surface of the forearm of the patient within the area 3 cm from the antecubital fossa until 5 cm from the wrist. A disposable blood lancet with drops of the allergen extract was used to lightly prick the skin. A 0.5 µL of each crude pollen extract, histamine as positive control, and 1X phosphate buffered solution (PBS) were placed 2 cm apart and skin was pricked using a disposable blood lancet. After 15-20 minutes, wheal sizes were measured. A wheal was considered positive if it was 3mm greater than the negative control. A copy of the results presented in Data Collection Form (Appendix A) were given to the patients.

Enzyme-Linked Immunosorbent Assay (ELISA)

The method was based on the protocol used by Dalmacio et al.[8] with some modifications. Pollen extracts at 100 µg/mL diluted in carbonate-bicarbonate buffer were coated overnight at 4 °C onto the wells of high-binding microtiter plates (Corning Costar). Plates were blocked with 1% non-fat dry milk in Phosphate buffered saline with Tween-20 (PBST) (0.01 M PBS, pH 7.4 with 0.05% Tween-20) for 2 hours at 37 °C. Serum samples, 1:5 (v/v) in dilution buffer (0.1% non-fat dry milk in PBST), were dispensed onto the wells and incubated for 1 hour at 37 °C. Plates were incubated with 1:1000 dilution of horseradish peroxidase (HRP)-conjugated anti-human IgE from Goat (Invitrogen A1870) for 1 hour at 37 °C. Washing was done after each step using PBST. Colorimetric reactions were performed using 3,3',5,5'-Tetramethylbenzidine and the reaction was stopped with 2 M Sulfuric acid. Absorbance was read at 450 nm using SPECTROstar Nano Microplate Reader. Spectrophotometric assays were done in triplicates. The mean absorbance readings were computed. The result is positive if the mean absorbance reading was above the cut-off value. Cut-off value was obtained from blanks using the formula by Frey et al.[9] described below.

$$\text{Cut-off value} = \text{Mean} + \text{SD}^*\text{f}$$

Where: Mean = mean absorbance of the blank, SD = standard deviation of the absorbance of the blank, f = multiplier dependent on the number of replicates at 95% confidence interval (Appendix B).

Statistical analysis

Data characteristics of test subjects and positive reactions to different pollen allergens using SPT and sIgE ELISA were presented as frequency (percentage). Pearson Correlation

was used to determine the effect of pollen purity and pollen concentration to the reactivity rates. Percent agreement of sensitization profile or proportion of patients with positive sIgE ELISA among those with positive SPT were graphically represented. Frequency tables and graphs were created using MS Excel and/or GraphPad Prism.

RESULTS

A total of 165 patients with respiratory allergies underwent SPT and among them, 76% had positive results to at least one pollen allergen. Blood was extracted from those patients with positive SPT results for serum specific IgE. Therefore, it is to be noted that these are not different groups of patients. The characteristics of the test subjects were shown in Table 1.

The median age of patients who tested positive in SPT and sIgE ELISA is 27 and 26 years old, respectively. There were more females in the study and most of them have allergic rhinitis alone or have allergic rhinitis with asthma.

As seen in Table 2, the purity of pollen samples ranged from 58.62% (talahib) to 96.06% (pigweed), while the total protein concentration of the pollen extracts ranged from 1.08 mg/mL (talahib) to 9.63 mg/mL (pigweed).

Looking at the positivity rate per pollen allergen (Table 2), there were more patients sensitized to weeds on SPT. For the sIgE ELISA, coconut had the highest sensitization rate followed by pigweed, Johnson grass, and rice. Talahib had the lowest sensitization rate for both SPT and sIgE ELISA.

Protein concentration and pollen purity values are presented as mean ±SE. Figure 1 shows that the highest positive agreement or the proportion of patients with positive sIgE ELISA among those with (+) SPT was highest with coconut (53.06%), followed by Johnson grass (31.25%), pigweed (30.67%), and rice (29.31%). Wild daisy had the lowest percent positive agreement (4.0%).

Majority of pollen sensitized patients on SPT are poly-sensitized. In contrast, the proportion of monosensitized patients is 44% and polysensitized is 55.7% on sIgE ELISA (Table 3).

Pearson correlation was performed between pollen purity/protein concentration and SPT/ELISA positivity rates. There was a strong positive relationship between pollen purity and ELISA positivity rate (r = 0.86, p-value = 0.0004), while a positive relationship was seen between protein concentration and SPT positivity rate (r = 0.58, p-value=0.0487). Positive correlation was observed, showing that as pollen purity increases, ELISA positivity increases and as protein concentration increases, SPT positivity rate increases.

Table 1. Baseline Characteristics of Patients with Positive Results

Characteristic	SPT(+) patients (n=125)	sIgE(+) patients (n=70)
Age group (years old)		
19-44	122 (97.6%)	70 (100.0%)
45-50	3 (2.4%)	0 (0.0%)
Median	27 yr	26 yr
Sex		
Male	38 (30.4%)	19 (27.1%)
Female	87 (69.6%)	51 (72.9%)
Disease		
Allergic Rhinitis	91 (72.8%)	49 (70.0%)
Asthma	3 (2.4%)	1 (1.4%)
Allergic Rhinitis + Asthma	31 (24.8%)	20 (28.6%)

DISCUSSION

It was found that more patients with allergic rhinitis were recruited in the study, mostly females, and in the 19-44 years of age. This was in agreement with the study by Sabit et al.[10] wherein allergic cases were observed more frequently among patients aged 20-40 years and among females.

Similar to the study by Navarro-Locsin and Lim-Jurado[1] that used allergen extracts outsourced from the USA, high sensitization rates were observed for Johnson grass, acacia,

Table 2. Proportion of Positive Results per Pollen Extract

Sources of pollen	Protein concentration (mg/mL)	Pollen purity (%)	Frequency (Percentage)	
			SPT (n=125)	sIgE ELISA (n=70)
Pigweed	9.63 ± 0.21	96.06 ± 1.00	75 (60.0%)	23 (32.8%)
Wild daisy	6.11 ± 0.06	73.45 ± 4.28	75 (60.0%)	3 (4.3%)
Makahiya	2.87 ± 0.08	94.85 ± 0.64	67 (53.6%)	16 (22.8%)
Johnson grass	4.51 ± 0.02	90.01 ± 2.73	64 (51.2%)	20 (28.6%)
Cogon	4.38 ± 0.03	91.23 ± 1.42	63 (50.4%)	14 (20.0%)
Mango	9.14 ± 0.24	69.07 ± 2.14	62 (49.6%)	8 (11.4%)
Foxtail	6.34 ± 0.02	83.33 ± 16.67	60 (48.0%)	12 (17.1%)
Acacia	4.37 ± 0.18	80.05 ± 4.14	59 (47.2%)	12 (17.1%)
Rice	5.51 ± 0.07	93.19 ± 1.19	58 (46.4%)	17 (24.3%)
Ipil-ipil	5.64 ± 0.02	66.79 ± 10.95	57 (45.6%)	7 (10.0%)
Coconut	4.33 ± 0.11	90.86 ± 3.87	49 (39.2%)	26 (37.1%)
Talahib	1.08 ± 0.01	58.62 ± 4.47	45 (36.0%)	3 (4.3%)

Table 3. Sensitization Patterns of Patients Positive in SPT and sIgE ELISA

	SPT (n=125)	sIgE ELISA (n=70)
Monosensitized	8 (6.4%)	31 (44.3%)
Grasses *Johnson grass, Rice, Cogon, Foxtail, Talahib*	1 (0.8%)	10 (1.4%)
Weeds *Pigweed, Makahiya, Wild daisy*	4 (3.2%)	10 (1.4%)
Trees *Coconut, Ipil-ipil, Acacia, Mango*	3 (2.4%)	11 (1.6%)
Polysensitized	117 (93.6%)	39 (55.7%)
Grasses only	6 (4.8%)	4 (5.7%)
Weeds only	-	1 (1.4%)
Trees only	-	-
Grasses and Weeds	22 (17.6%)	7 (10.0%)
Grasses and Trees	8 (6.4%)	10 (14.3%)
Weeds and Trees	6 (4.8%)	6 (8.6%)
Grasses, Weeds, and Trees	75 (60.0%)	11 (15.7%)

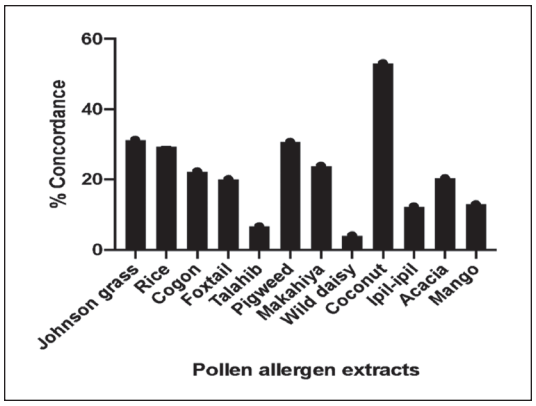

Figure 1. Positive agreement or proportion of patients with positive sIgE ELISA among those with (+) SPT.

and pigweed (57.9%-58.7% vs. 47.2%-60.0%). In addition, using local allergen extracts, it was found that generally there were more patients sensitized to weeds on SPT. Noticeably, IgE sensitization was found to be higher with the SPT compared to the sIgE ELISA.

SPT is regarded as the most effective diagnostic test to detect IgE-mediated type I allergic reactions like allergic rhinitis and asthma. Its reactivity depends on an intact immune system and the presence of IgE sensitized mast cells that release mediators particularly histamine which produces the wheal and flare reaction.[11] SPT involves introducing controlled amounts of allergen and control substances into the skin. It is generally safe, has fast results, cost-effective, and convenient. However, some patients have dermatographism or extensive skin lesions or cannot be put off antihistamines and thus, specific IgE tests which measure specific IgE in the serum of patients are done instead. Specific IgE tests using ELISA are dependent on the IgE levels in patient's sera and in the unpredictable mixture of allergenic and non-allergenic substances in crude extracts. In this study, the highest positive agreement between sIgE ELISA and SPT was only 53.06% with the lowest at 4.0%. Discrepancies between SPT and ELISA results are evident. These are probably due to the low IgE levels in sera of the patients and thus undetectable or low binding between the sera IgE and the allergen coated in the polystyrene plates. It must be kept in mind that a positive reaction from SPT is mainly due to mast cell-bound IgE, whereas positive reaction from ELISA is due to circulating IgE which becomes present only after the saturation of the whole receptor system of the organism.[6] Cabauatan et al.[12] further made an assumption that tropical flora produces highly glycosylated allergens and these non-allergenic carbohydrate motifs, masking the allergenic protein allergens, instead react with IgE antibodies without inducing relevant clinical symptoms. SPT and serum-specific IgE ELISA results are not interchangeable as evident in this study and as stated in the position paper on the practical guide to skin prick tests in allergy to aeroallergens.[13] Instead, they may be considered as complementary to one another.[14] Essentially, results of both SPT and ELISA must be correlated with the patient's clinical history to effectively diagnose allergic rhinitis and decide on allergen immunotherapy prescription in atopic patients.

This study showed that most of the patients were polysensitized. Our study had a higher polysensitization rate at 93.6% and our study population were mostly adults. Polysensitization can be due to the presence of cross-reactivity of the allergens where the same IgE binds to several different allergens with common structural features. This could also be due to co-sensitization where different IgEs bind to allergens that may not necessarily have common structural features.[15] In a review by Migueres et al.[15], between 50% and 80% of patients consulting allergists are polysensitized. Furthermore, polysensitization rate depends on the population (more adults were polysensitized than children) and region. Similar to our findings, it was also reported that in the Mediterranean area, patients with pollen-induced allergic rhinitis are often polysensitised.[16]

In this study, pollen purity was defined as the percentage of intact target pollen grain in the collected sample; whereas protein concentration is the protein content in the pollen after extraction with PBS. The purity of pollen samples varied from 58.62% for talahib to 96.06% for pigweed. This variation may have been caused by different methods of collection used, depending on what is feasible to the type of inflorescence as well as the pollen type. Furthermore, some of the pollen samples were collected near pavements prone to contamination from dusts and other plant species which are difficult to separate from the target pollen due to having similar sizes. The total protein concentration of the pollen extracts ranged from 1.08 mg/mL (talahib) to 9.63 mg/mL (pigweed). Although the same extraction method and protein content determination assay were done for all samples, the innate variation of protein content per plant pollen results in different protein concentration. Pearson

correlation showed that as protein concentration increases, SPT positivity rate increases. Allergens are generally proteins or glycoproteins. The higher protein content in the extract may mean higher concentrations of the allergenic protein, hence the higher sensitivity and reactivity of patients to the test panel. This is the reason why allergenic extracts should, ideally, be standardized where the major allergenic proteins are quantified to ensure reliability and reproducibility of skin test results. Protein extracts lose their potency or allergenicity when protein is acted upon by proteolytic enzymes or denatured due to improper storage conditions.[17] As expected, positive correlation was observed for pollen purity and ELISA positivity. With high percent pollen purity, less protein from contaminants will compete for binding to the polystyrene plates. Target allergenic proteins will have a high coverage of the plate wells in which the_specific IgE will bind to, resulting in a reading above the titer. This is similar to the principle during purification of specific antisera, where the antibodies are captured by highly pure antigens bound to a surface or beads. The purity of antigen is crucial for generation of specific antisera, where generally no more than 1%-2% of contaminants should be present.[18]

CONCLUSION

Pollen allergens from grasses, weeds, and trees locally found in the Philippines yielded positive reactivities for both SPT and sIgE ELISA tests, with high sensitization rates being observed among the grasses and weeds, especially pigweed and Johnson grass. Majority of pollen sensitized patients on SPT are polysensitized. Discrepancies on the frequencies of positive results between SPT and ELISA were observed which may be attributed to the low serum IgE levels and low quantities of effectual allergen components in the extracts. Results of both SPT and ELISA must be correlated with the patient's clinical history, particularly the patient's exposures, and physical examination.

Disclaimers

This paper represents the opinions of the authors, and is the product of professional research. It is not meant to represent the position or opinions of the University of the Philippines Manila or Philippine Council for Health Research and Development, nor the official position of any staff members. Any errors are the fault of the authors.

Statement of Authorship

All authors certified fulfillment of ICMJE authorship criteria.

Author Disclosure

The authors declare that they have no relevant or material financial interests that relate to the research described in this paper.

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APPENDICES

POLLEN STUDY DATA COLLECTION FORM

Name:		Subject no.:	
	Last First Middle	PGH Case No.	
Age/Sex:		Birthdate (mm/dd/yyyy): / /	
Address:			
Mobile no.:		Diagnosis: ☐ Allergic Rhinitis	☐ Bronchial Asthma
Landline:			☐ Allergic Rhinitis & Bronchial Asthma

Skin Test to Pollens Date: _____

Medications Taken	Date of Last Intake

	Result (mm x mm)	Interpretation (encircle if +)
A. Weeds		
1. *Mimosa pudica* (makahiya)	__x__	+
2. *Amaranthus spinosus* (pigweed)	__x__	+
3. *Tridax procumbens* (wild daisy)	__x__	+
B. Grasses		
1. *Imperata cylindrical* (cogon)	__x__	+
2. *Pennisetum polystachion* (foxtail) – Los Banos	__x__	+
3. *Pennisetum polystachion* (foxtail) - Pangasinan	__x__	+
4. *Sorghum halapense* (Johnson grass)	__x__	+
5. *Oryza sativa* (rice pollen)	__x__	+
6. *Saccharum spontaneum* (talahib)	__x__	+
C. Trees		
1. *Samanea saman* (acacia)	__x__	+
2. *Cocos nucifera* (coconut)	__x__	+
4. *Leucaena leukocephala* (ipil-ipil)	__x__	+
5. *Mangifera indica* (mango)	__x__	+
Histamine (1 mg/ml)	__x__	
Glycerine (50% in PBS)	__x__	

Done by: _____

Appendix A. Pollen Study Data Collection Form.

Standard deviation multipliers (f) for calculation of cutoffs[9]

Number of Controls (n)	Confidence level (1 - α)				
	95.0%	97.5%	99.0%	99.5%	99.9%
2	7.733	15.562	38.973	77.962	389.823
3	3.372	4.968	8.042	11.460	25.783
4	2.631	3.558	5.077	6.530	11.420
5	2.335	3.041	4.105	5.044	7.858
6	2.177	2.777	3.635	4.355	6.366
7	2.077	2.616	3.360	3.963	5.567
8	2.010	2.508	3.180	3.712	5.076
9	1.960	2.431	3.053	3.537	4.744
10	1.923	2.373	2.959	3.408	4.507
11	1.893	2.327	2.887	3.310	4.328
12	1.869	2.291	2.829	3.233	4.189
13	1.850	2.261	2.782	3.170	4.078
14	1.833	2.236	2.743	3.118	3.987
15	1.819	2.215	2.711	3.074	3.912
16	1.807	2.197	2.683	3.037	3.848
17	1.797	2.181	2.658	3.005	3.793
18	1.787	2.168	2.637	2.978	3.746
19	1.779	2.156	2.619	2.953	3.704
20	1.772	2.145	2.602	2.932	3.668
25	1.745	2.105	2.542	2.852	3.535
30	1.727	2.079	2.503	2.802	3.452

α = significance level. Standard deviation multipliers are derived from the critical values for a one-tailed *t*-distribution (for a comprehensive listing of critical values for a *t*-distribution see Federighi, 1959).

Appendix B. Standard deviation multipliers (f) for calculation of cutoffs.