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Amaranthus Pollen Allergens: Protein Diversity and Impact on Allergy Diagnosis

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Abstract

Allergenic weeds dominate the pollen air flora (> 80%) of Saudi Arabian environment. Two species viz *Amaranthus viridis* (Av) (Slender or Green *Amaranth*) and *A. lividus* (Al) are the most prevalent components of this flora. Although all *Amaranthus* species appear to be allergenic and a potential cause of respiratory allergy, neither a diagnostic extract nor raw pollen grains for Av is available commercially for comparative study on patients.

Thus, in order to determine IgE mediated sensitization of Av and to observe cross-reactivity patterns with other species, an allergological study was conducted using seven *Amaranthus* species. Row pollen of 5 *Amaranthus* species were acquired from two commercial sources (Greer USA, Allergon Europe) while Av & Al pollen was collected indigenously. Allergenic extracts were prepared using pollen grains from both sources in buffered saline. Skin Prick Test (SPT) was conducted on 132 allergic patients using all seven extracts.

Protein separation of all seven *Amaranthus* species was conducted by SDS-PAGE. The results indicate that the species of *Amaranthus* vary in their protein profiles with a pattern of cross SPT reactivity between the species. However, as the exposure takes place with prevalent pollen form Av and Al, the commercial extracts using species not present in the region may not be fully relevant to the patients for diagnosis and immunotherapy.

Keywords: Allergy; Pollen; *Amaranthus viridis*; Diagnosis; *Amaranthus* species; Protein diversity

Abbreviations

SDS-PAGE: Sodium Dodecyl Sulphate Polyacrylamide Electrophoresis; AV: *Amaranthus viridis*; SPT: Skin Prick Test; RAC: Research Advice Council

Introduction

Allergy and Asthma in both children and adult can be caused by many allergenic pollen grains from weeds, trees and grasses [1]. World allergenic pollen flora varies in their nature and quantity from place to place and fluctuates with geography and climate.Bronchial Asthma is very common allergic disease occurring in all age groups, particularly children, all over the world and the trend of asthma prevalence in both developed and developing countries are increasing over the last 30 years [2].

Environmental factors are known to play an important role in the elicitation of asthma in genetically predisposed individuals. Although there has also been an increase in the awareness among doctors to diagnose asthma, a combination of various other factors may also be involved in the increased prevalence of asthma [3]. The soil and climate of KSA was once considered unfavorable for plant growth. A large number of plants have been introduced to the kingdom in recent years [4].

The genus *Amaranthus* consist of several species. It is an allergenic weed shedding pollen in the air throughout the year in Saudi Arabia with peaks in autumn months. There are a number of *Amaranthus* species in Saudi Arabia as listed in (Table 1). Each of them, with some synonym, is known by a common name as well. Both, the common and synonymic names are also presented in this table. However, the dominant species on the ground and frequently encountered pollen in the air belongs to *A. viridis* [5] (Figure 1).

Survey of the *Amaranthus* species was made in the deserted countryside of Saudi Arabia in view to observe the growth pattern and collection. It was revealed that there are some species, which are very close to *Amaranthus* but appeared not to be *Amarathus viridis*.

The allergenic extract, prepared using *Amaranthus* pollen, are used for the diagnosis of respiratory allergic disorder to this pollen and like-wise, the treatment vaccine for immunotherapy is prepared using pollen extract.

It appears that *A. viridis* extract is not included in the diagnostic profile in Saudi Arabia by the clinicians and instead, unrelated imported/commercial extract of other *Amaranthus* spp. under the common name of Pigweeds are included. This is likely to result in false negative reactivity in those patients who are exposed to *A. viridis*. There are only up to 30% cross-reactivity within the weeds pollen allergy but no such cross-reactivity has been documented within all *Amaranthus* pollen allergens [6]. Apart from the cross reactivity, treatment by immunotherapy may not be successful unless precise molecular relation between offending allergen and desensitized allergens are established.

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Table 1: *Amaranthus* Species in Saudi Arabia [7-11].

Scientific name	Synonym	Common name
Amaranthus albus	*A. var. pubescens, A. graecizans auct. non, A. var. pubescens Uline & Bray* [7]	White Pigweed, Prostate Pigweed, Pigweed *Amaranth*, White *Amaranth*.
A. caudatus	*A. edulis Speg. Amaranthus leucocarpus* (S.Watson.), [8]	Pendant *Amaranth*, Love-lies-Bleeding, Tassel Flower, Quilete.
A. graecizans ssp sylvestris	*A.graecizans* [9,10] *A.blitum*	
A. graecizans	*A. angustifolius A.albus* [9,10]	Prostate Pigweed
A. hybridus ssp hybridus	*A. chlorostachys* [10].	Smooth *Amaranth*, Smooth Pigweed, Red *Amaranth*, Slim *Amaranth*.
A. hybridus ssp crutentus	*A. crutentus* [10].	Purple A., aka, Red A, Mexican grain A, Caterpillar.
Amaranthus palmeri	[11]	Palmer's *amaranth*, palmer pigweed, careless weed
A. lividus **	*A. blitum, A. ascendens* [10].	Purple *Amaranth*
A. spinosus	[9,10]	Spiny *Amaranth*, Prickly *Amaranth*, Thorny *Amaranth*, Spiny *Amaranthus*.
Amaranthus tricolor	*A. tristis, A. mangostanus* [10]	Joseph's-coat
A. viridis *	*A. gracilis Desf.* [8]	Slender *Amaranth*, Green *Amaranth*

***Most common in Saudi Arabia (+++) (as per growth pattern)
****Less common in Saudi Arabia (++) (as per growth pattern)
All others species are rare and sporadic.

Figure 1: *Amaranthus viridis* weed.

Materials and Methods

Collection of indigenous *Amaranthus*

Two *Amaranthus* species (Av, Al) were primarily collected from Riyadh, Jeddah, Taif and Najran regions. Majority of these species were found growing in parklands, home backyard, home gardens, lawns etc.

Several lots of flowering *Amaranthus* were collected at different time intervals from different places. All collections were properly dried. After drying the collected plants, anthers were separated. The separated anthers were further dried, treated and teased with acetone and 95% alcohol, centrifuged and dried as raw material, stored at 4°C and used in the preparation of extracts. Pollen samples showing more than 90% purity were included in the investigations.

Pollen from commercial sources

Based on the international availability, commercial pollen grains of the following species were purchased from various commercial suppliers in Europe and USA:

These included: *Amaranthus palmeri*, *Amaranthus tuberculatus*, *Amaranthus retroflexus*, *Amaranthu shybridus* (Greer Laboratory, USA), (*Amaranthus retroflexus*, *Amaranthus tamariscinus*. Allergon Company, Europe) [7-11].

Pollen protein extraction

Both collected and commercial pollens were defatted with excess of diethyl ether / n-butanol to achieve maximum removal of lipids and pigments. Antigenic protein was extracted from the defatted pollen with 1:10 weight per volume (w/v) concentration. The extract was prepared in Phosphate Buffered Saline [12] (10 mM PBS pH 8 at 40°C for 72 hrs). After the extraction, it was centrifuged at 4000 rpm for 15 min and the supernatant was dialyzed (mol. wt. cut limit: 3500) exhaustively against 85 % PBS, lyophilized by freeze drying system in small aliquots and stored at –200°C and reconstituted, when and as required. Protein content of each extract was determined by Bradford method [13]. The extracts were sterilized by bacterial filter by passing through 0.45 mm and 0.22 mm filter using Millipore filter units. For in vivo SPT, 50% glycerinated extracts were prepared. The purity and sterility for each extract was tested using Brain Heart Infusion Agar and Blood Agar for at least 15 days at 37°C. The test was negative indicating no contamination.

Sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE)

The procedure outlined by Laemmli [14] was followed. SDS-PAGE was carried out using 12 % polyacrylamide gel using Mini Electrophoretic Apparatus (Bio Rad). Extracts with varying protein concentrations were used in loading. The gels were calibrated with marker proteins with molecular weights of 10, 15, 20, 25, 37, 50, 75, 100, 150, 250 kD (Bio-Rad). The gels were stained using staining solution (10% glacial acetic acid, 0.25 % Commassie brilliant Blue in 45% methanol), then destained for varied periods until protein bands appeared clear. After destaining, the gels were scanned.

Skin Prick Test (SPT)

Skin prick tests were performed on 39 allergic patients and 10 healthy control subjects, attending the Allergy clinic at King Khalid University Hospital, Riyadh. Phosphate buffered saline and histamine were also tested as negative and positive control respectively. The skin response was observed after 15-20 minutes of the test and graded as per the criteria:

<3mm Negative,

≥3mm Low Positive,

5-10mm Moderate Positive, and

>10mm Strong Positive

Serum samples

Venous blood was drawn from 17 skin test positive patients and sera was separated by centrifugation and stored at -20°C in small aliquots for further use. Blood samples from 10 healthy volunteers were also collected to act as control. ARAC approved consent form was signed by each patient for SPT and blood draw.

Immunoblot

Electrophoretic transfer of proteins to PVDF membrane following the method of Towbin et al. [15]. Proteins separated by SDS-PAGE were electrophoretically transferred to a 0.45µm Polyvinylidene Difluoride (PVDF) membrane for immune detection of IgE in serum of sensitized subjects bound to allergenic proteins. Highly positive sera from hypersensitive patients were used to determine the IgE binding fractions in pollen extracts.

PVDF membrane (0.45 µm) of the size of the gel was soaked in the transfer buffer, Tris-glycine buffer (25mM Tris, 200 mM Glycine, 20% methanol, pH8.3) an hour before the transfer of proteins. Proteins were then blotted to membrane by electro transfer using the transfer buffer at 30mA at 4°C for overnight.

The un-reacted sights on the membrane were blocked with 5% non fat milk in 0.05 % Tween20 Phosphate Buffered Saline (PBST) at room temperature for 1 hour. Washed by PBST then, membrane is incubated with pooled sera of positive individual. Pooled sera from healthy individual showing negative skin reactivity were used as control.

In all incubations serum was diluted in the ratio of 1:500 using PBS containing 0.05 % Tween20. Membrane was washed thoroughly using 0.05% PBST. After washing the membrane was blocked by non fat milk (5%). Membrane was incubated with antihuman IgE peroxidase conjugate (Sigma) in the ratio of 1:10000 in 0.05% PBST for I hour at room temperature. The membrane then washed thoroughly 4 times by washing buffer 0.05% PBST. After the last wash the membrane was washed by PBS to remove all the Tween. Membrane was developed in dark room after ECL super signal incubation for 5 minutes.

Results

Protein estimation

The Protein concentration for each sample was:

1. *Amaranthus viridis* – 1.172 µg/µl

2. *Amaranthus lividus* – 0.637 µg/µl

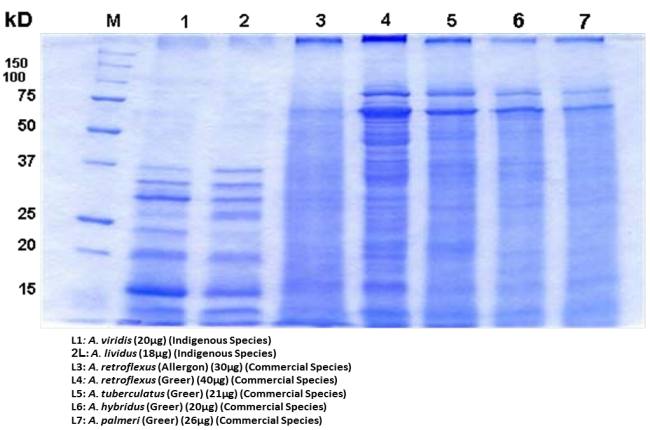


Figure 2: 12% SDS-PAGE of different *Amaranthus* Species.

3. *Amaranthus retroflexus* (Allergon) – 1.598 µg/µl

4. *Amaranthus retroflexus* (Greer) – 2.379 µg/µl

5. *Amaranthus tuberculatus* (Greer) – 0.852 µg/µl

6. *Amaranthus hybridus* (Greer) – 0.941 µg/µl

7. *Amaranthus palmeri*(Greer) – 1.300 µg/µl

Sodium Dodecyl Sulphate Polyacrylamide Electrophoresis (SDS-PAGE)

Figure 2 - A 12 % SDS-PAGE, shows equal volumes of samples were loaded, the highest concentration was in L4 (*Amaranthus retroflexus*) from Greer company which means that the purity of the pollens were 100%.

Remarkable differences in protein profile between the indigenous species (L_1, L_2) and the commercial ones (L_3- L_7) are shown in the gel. The indigenous samples showed bands at lower molecular weight ranging between 36 kD& 14 kD, while the commercial species showed proteins at higher ones. The indigenous *Amaranthus viridis* has bands at 36, 33, 31, 24, 20 and 15 kD, and *Amaranthus lividusat* 36, 33, 31, 29, 20 and 15 kD. The commercial ones from Greer *Amaranthus retroflexus* showed bands at 87, 70, 48, 40, 36, 32, 28, 26, 20, 17 and 14 kD. *Amaranthus tuberculatus* at 87, 70, 40, 36, 24 and 17 kD. *Amaranthus hybridus* at 87, 70 and 40 kD. *Amaranthus palmeri* at 87 and 70 kD.

SPT results

Out of 132 consecutive patients attending the Allergy clinic at King Khalid University Hospital, Riyadh (KKUH), sixty five patients (65/47.1%) reacted positively to *Amaranthus* extracts, both commercial and locally prepared. The skin test reactivity of the 39 patients, who participated in the study are presented in (Table 2 and Figure 3).

In the whole group (39), 31(76.92%) reacted to *A. viridis* (indigenous species), while 26 patients (66.66%) reacted to *A. lividus* (indigenous species).

Twenty five patients (64.10%) reacted to both local extracts. Four patients showed strong reactions to the local *Amaranthus* allergens.

Reactions to the other *Amaranthus* species were as follows:

Table 2: Skin test reactivity of 39 patients to seven *Amaranthus* extracts.

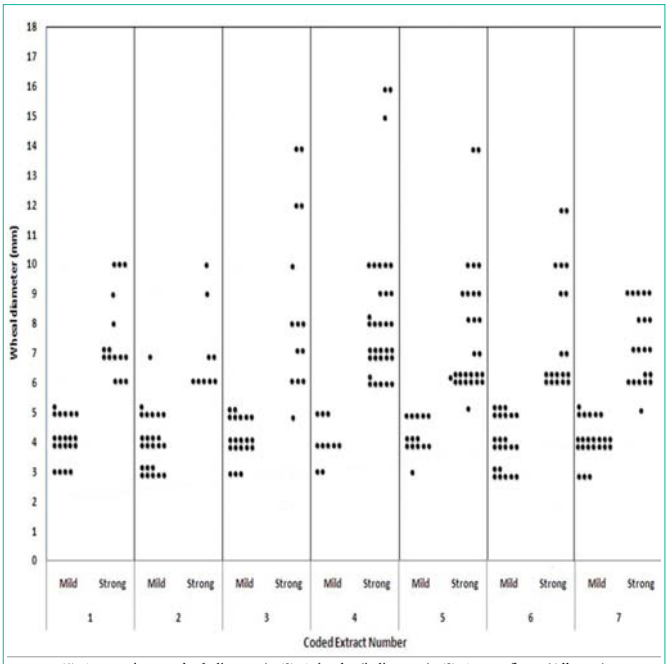
	*(wheal diameter in mm)						
ALLERGENS Patient No.	1 *A. viridi* (local)*	2 *A.lividus* (local)*	3 *A. retroflexus* (Allergon)*	4 *A. retroflexus* (Greer)*	5 *A. tuberculatus* (Greer)*	6 *A. hybridus* (Greer)*	7 *A. palmeri* (Greer)*
1	3	4	7	6	6	7	7
2	4	-	-	4	-	-	-
3	8	7	7	5	9	5	8
4	5	5	4	10	6	4	4
5	7	-	8	10	8	7	6
6	4	-	-	7	4	4	4
7	6	7	-	-	-	-	-
8	-	-	4	7	4	4	4
9	-	-	-	4	5	-	6
10	3	3	5	8	10	6	5
11	5	-	6	5	5	6	6
12	7	5	4	8	7	4	7
13	10	10	14	10	10	10	9
14	5	3	8	9	7	6	5
15	4	3	5	7	6	6	5
16	-	-	-	-	4	3	4
17	-	-	3	5	5	5	4
18	5	4	8	15	10	12	8
19	4	4	6	9	6	5	6
20	-	-	-	6	4	4	3
21	5	4	4	4	4	4	4
22	6	6	14	9	9	12	8
23	9	8	5	7	6	5	5
24	4	5	10	6	5	5	7
25	-	-	-	7	5	3	4
26	3	3	3	7	4	6	5
27	4	-	5	3	-	4	6
28	4	4	6	8	6	6	7
29	5	4	7	8	6	5	6
30	3	-	-	5	3	3	3
31	-	4	5	6	8	6	4
32	10	6	4	16	6	3	4
33	7	6	4	8	9	9	9
34	7	5	12	10	14	10	9
35	4	3	5	7	6	6	5
36	4	3	4	7	6	3	4
37	-	-	3	6	5	5	6
38	6	4	-	4	4	5	3
39	7	5	5	4	4	4	4

30(76.92%) to *A. retroflexus* (Allergon), 37(94.87%) to *A. retroflexus* (Greer), 36(92.30%) to *A. tuberculatus* (Greer), 36(92.30%) to *A. hybridus* (Greer) and 37(94.87%) to *A. palmeri* (Greer).

Immunoblot

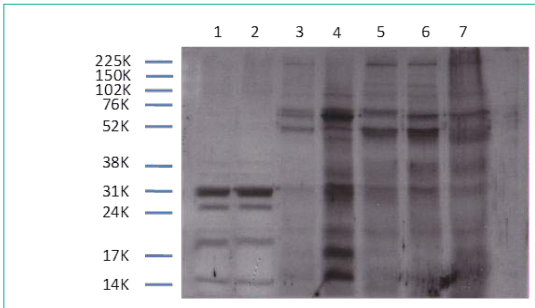
1. Immunobloting of serum samples (17 patients), we found that 94% of the *Amaranthus* sensitized (IgE mediated positive SPT) individuals have IgE-binding antibodies to *Amaranthus viridis* (Indigenous) pollen extract. The major *Amaranthus* allergen defined as binding IgE from most subjects is 52, 31 Kda. Other IgE-binding allergens were found at 38, 20, 17and 14Kda. 3.

2. All patients reacted to proteins at 31 Kda and 52 Kda. It was interesting to note that indigenous extracts contained 31 Kda & 52



(1) *Amaranthus viridis* (indigenous), **(2)** *A. lividus* (indigenous), **(3)** *A. retroflexus* (Allergon), **(4)** *A. retroflexus* (Greer), **(5)** *A. tuberculatus* (Greer), **(6)** *A. hybridus* (Greer), **(7)** *A. palmeri* (Greer).

Figure 3: Mild and Strong SPT reactivities of various *Amaranthus* extracts.



1-Amarnthus viridis (Indigenous),2-A.lividus. (Indigenous),3-A.retroflexus.(Allergon), 4-A.retroflexus (Greer)5-A.tuberculatus. (Greer),6A.hybridus. (Greer) ,7-A.palmeri. (Greer)

Figure 4: Immunoblot showing *Amaranthus* species specific IgE binding fractions of antigenic extracts probed with the sera of SPT positive patient (p13).

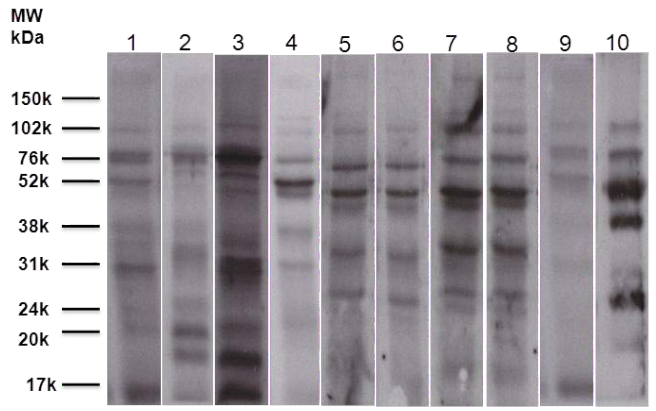


Figure 5: Individual sensitivity patterns to *Amaranthus retroflexus* extract on immunobloting.

Kda proteins and out of 17 patients, 16 (94%) reacted to indigenous extract (*A. viridis*). However, one patient who did not react to indigenous (*A. viridis*, possibly an error) reacted to 31 Kda protein of other indigenous (*A. lividus*). Likewise, two patients, who did not react to *A. lividus* (patient no. 6&7), reacted to *A. viridis*. Therefore, it was 100% immune-reactivity towards two species of indigenous *Amaranthus* species (Figure 4).

3. Surprisingly, all patients showed allergenicity to sample no.4 (Figure 5) and 76.47 % showed allergenicity to sample no. 3, species which are not found in KSA.

Discussion

This study has provided much important information as regards to *Amaranthus* allergens that are prevalent in the Kingdom of Saudi Arabia and those which are imported into the country for diagnostic and therapeutic reasons.

There are only a handful of companies in the world, mainly in Europe and North America, producing *Amaranthus* extract for diagnostic and therapeutic use. Most imported extract belong to species which are not found in the Kingdom of Saudi Arabia. The list of commercial Allergenic Pollen Powder (& aqueous extracts) revealed that none of them produce extracts for SPT using *A. viridis*. Our literature search also indicates that there are no commercial suppliers of *A. viridis* pollen powder and extract. This is an interesting observation because *A. viridis* is the dominant species in Saudi Arabia while no suppliers has access to this pollen in the world market to date.

"*Amaranthus* extract" means extract from any species or variety of *Amaranthus* which may or may not include the *viridis* species. Some of the *Amaranthus* are known as: *Amaranthus lividus* (Purple *amaranth*), *Amaranthus palmeri* (Careless weed), *Amaranthus retroflexus* (Pigweed, Rough (Redroot)), *Amaranthus viridis* (Slender *amaranth*, Green *amaranth*) etc.

There may be clinics and hospitals in the Kingdom getting commercial "*Amaranthus* extract", but the question is that they need to know whether the species they are using is available in Saudi Arabian environment and how prevalent they are? Are patient exposed to the same species where they live??

The result has indicated that there is cross-reactivity between some species of *Amaranthus* and that is the reason that *A. retroflexus*, a species not found in Saudi Arabia, reacted in most patients [16]. The *A. retroflexus* was purchased from Greer company in the United States was highly purified. The allergen extract prepared in our Lab using the same technique, as used for others, gave a high protein content compared to others. However, when the main allergen in our environment is identified, it is questionable to use cross-reactive allergens [17].

It has been noted (personal communication with many Allergists in the Kingdom) that patients undergoing immunotherapy with "Pollen allergens" are not successfully treated. The probable reason may be the precise molecular relationship to desensitize the patient and the causative allergenic determinants may be different from the determinants in immunotherapy products. In the present study, we

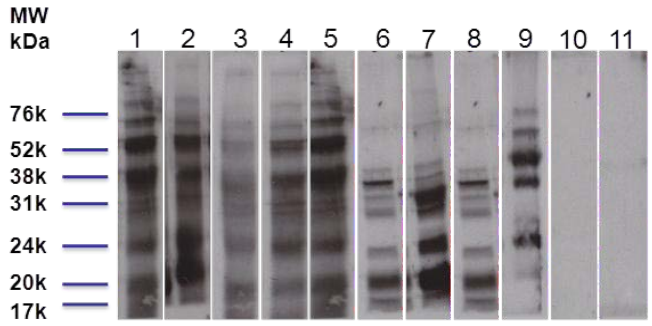


Figure 6: Immunoblot showing *Amaranthus viridis* specific IgE binding fractions of antigenic extracts when probed with the sera of positive patients [1-9] and control sera [10,11].

found a high degree of reactivity to *Amaranthus viridis* with their IgE binding allergenic proteins at 31 Kda and 52 Kda (Figure 6). Some of the commercial extract also contained the same allergenic proteins [18].

Our study also revealed that though there are a good number of individual who are SPT positive but in immunoblot, even a higher degree of reactivity was recorded.

Conclusion

We therefore conclude that despite cross reactivity within *Amaranthus* species efforts should be made to use Av or Al extracts for diagnosis and treatment especially patients in Saudi Arabia.

Acknowledgment

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