

Cultivar-Specific IgE-Epitopes in Date (*Phoenix dactylifera* L.) Fruit Allergy

Correlation of Skin Test Reactivity and IgE-Binding Properties in Selecting Date Cultivars for Allergen Standardization

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Key Words

Cultivars · Date fruits · ELISA · Epitopes · IgE · Immunoblotting · Major allergens · Standardization

Abstract

Background: Date fruits are allergenic and standardized extracts are required for diagnosis and therapy of this allergy. Since there are several cultivars of dates, this study was carried out to assess the allergenicity of different cultivars in order to select suitable source material for standardization. **Methods:** The protein profiles of 18 of the most commonly sold varieties were compared by SDS-PAGE and their relative allergenicity assessed by SPT and IgE-based ELISA and immunoblotting. Thirty-two date fruit-sensitive patients were skin tested with a pooled extract from all the cultivars. Six of the patients with high SPT results ($\geq 3+$) who volunteered were further tested with the 18 cultivars and their sera used in ELISA and immunoblotting. **Results:** Six of the cultivars gave high SPT-positive reactions in ≥ 4 of patients. Five of these high SPT-reactive cultivars gave high IgE ELISA scores (≥ 0.58) but individual cultivars varied in their number of IgE immunoblot bands. Cultivar-specific IgE-binding patterns indicated that only certain cultivars bound IgE at molecular weights of ≤ 14.3 and 27–33 kDa whilst all cultivars bound to a 54–58 kDa doublet. Cultivars

that bind to the ≤ 14.3 and 27–33 kDa bands appeared to form the majority of the high SPT-reactive cultivars. When individual sera of 24 of the 32 SPT-positive patients were used in IgE immunoblots with the pooled cultivar extract, all sera bound IgE at ≤ 14.3 and 27–33 kDa and about 60% of sera bound to a 54–58 kDa doublet bands. **Conclusions:** These results indicate that allergenicity of date fruits is a cultivar-specific phenomenon. Sixty to 100% of sera from date fruit-allergic patients bind IgE to three major allergens of ≤ 14.3 , 27–33 and 54–58 kDa. Five of the cultivars that evoke high SPT reactions, high IgE ELISA scores and bind IgE to the major allergens, can be selected for the preparation of 'in-house' allergen extracts and for allergen standardization.

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Introduction

Date fruits are allergenic and can elicit typical IgE-mediated symptoms in susceptible individuals [1, 2]. Antigens and allergens of date fruits cross-react with date pollen allergens and date fruit-sensitive as well as date pollen-allergic patients' sera recognize the same group of date fruit IgE-binding components [1]. In a previous investigation of the allergenicity of date palm pollen, we

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showed that pollen extracts from different cultivars were differentially allergenic [3]. However, only 3 out of the 10 cultivars studied possessed high IgE-binding qualities and also evoked high SPT reaction. The study also revealed that the antigenicity and allergenicity of date pollen is a cultivar-specific phenomenon rather than a species-specific phenomenon which was governed by the number, quantities or both, of the major IgE-binding components possessed by the cultivar under study [3].

There are over 100 cultivars of date fruits in the Riyadh area of Saudi Arabia alone. We hypothesized that if pollen, the fecundating organs of date palm cultivars are differentially allergenic, it is likely that the fruits which are their fertilization products would be equally heterogeneous in their allergenicity. The aim of this study was to evaluate fruits from 18 of the most commonly eaten cultivars for their SPT reactivity and IgE-binding properties as determined by ELISA and immunoblots. Results of this study would help facilitate the identification and selection of suitable cultivars for the preparation of in-house extracts and allergen standardization and also help identify the major allergenic peptides of dates [4].

Material and Methods

Reagents used in these experiments were of analytical grade and were purchased from BDH (Poole, Dorset, UK), Sigma Chemical Co. (St. Louis, Mo., USA), Fisher Scientific (N.J., USA) or as stated in the text. Eighteen of the most commonly eaten date fruit cultivars were obtained from a dates merchant (Kingdom Dates, Riyadh, Saudi Arabia). Date cultivars were purchased from one source to ensure that there were no allergenic variabilities due to treatment and handling since it is known that a number of factors like the degree of ripeness, time of harvest, methods of storage and post-harvest treatment can influence the allergen content of an extract. All cultivars were labelled and photographed to ensure easy identification. Fruits were surface sterilized, washed, weighed and extracted in phosphate-buffered saline (PBS) pH 7.8 [1, 5]. Extracts were extensively dialyzed, freeze dried, desalted and then reconstituted in double distilled deionized water as described previously [1]. The protein [6] and carbohydrate [7] contents were determined and all extracts were adjusted to the same protein concentration. Equal amounts from each cultivar extract were pooled, concentrated and evaluated in experiments to make sure that the extract mixture was antigenic and capable of evoking SPT reactions [3].

Skin Prick Test

The reactivity of the extract, pooled from all cultivars, was assessed in 32 date fruit SPT-positive patients with a mean age of 25 years and a population male:female sex ratio of 55:45. SPT was carried out using Morrow Brown needles. The reactivity of the subjects was demonstrated by a positive response to histamine HCl (1 mg/ml) and negative response to 50% glycerol in PBS. Wheals in an individual were scored on a scale of 0, 1+, 2+, 3+ and 4+. A histamine wheal

in an individual was assigned a value of 3+. An SPT reaction which when compared with histamine gave a wheal that is twice the size or has pseudopods was scored 4+. A wheal that is equal to that of histamine control was scored 3+, half that size was assigned a score of 2+ and any size less than half assigned 1+. If there is no visible reaction or a wheal was equal to negative control it was scored 0 (negative). All the patients gave positive results of varying degrees and 6 of them volunteered for further SPT evaluation with the 18 cultivar extracts. Skin prick tests were performed on the volar forearm with each of the cultivar extracts (1/20, w/v) in PBS (pH 7.4) containing 50% glycerol in PBS. 50% glycerol in PBS and histamine HCl (1 mg/ml) were also tested at the same time as negative and positive controls respectively as detailed above. Reactions were read after 15 min and the degree of reactivity of each cultivar recorded [1, 3].

Sera and Total IgE Estimation

Sera were prepared from blood collected from patients and healthy volunteers and the immunoglobulin fraction was collected as before, according to the method of Harboe and Ingliid [8]. The total IgE content of all sera were estimated by the Phadezyme PRIST (Pharmacia Diagnostics AB, Uppsala, Sweden) method following the manufacturer's guidelines.

Enzyme-Linked Immunosorbent Assay (ELISA)

The indirect method of Voller et al. [9] was used to assess and compare the reactivity of the cultivar extracts. Microtiter ELISA plates were coated with replicates of each of the cultivar extracts as well as the pooled extract (18 cultivars) at total protein concentrations of 50 µg/ml (100 µl/well) in coating buffer (0.05 M carbonate buffer, pH 9.6). Pooled sera from the 6 atopic-positive patients and pooled normal human sera were added after washes. All subsequent steps and all statistical analyses were carried out as described previously with appropriate controls [1, 3]. Briefly the scores obtained from these assays were calculated by comparison to mean and SD for all the various controls and of replicates. A Dynatech MR 600 automatic Microplate reader (Dynatech Laboratories Inc.) interfaced with an IBM computer and specifically programmed to calculate the mean and SD was used. Results were considered positive only if an ELISA score was 2 SD or higher than mean of all controls [1, 3].

SDS-PAGE and Immunoblotting

Cultivar extracts, adjusted to the same protein concentrations as described above, were separated in duplicate by SDS-PAGE using 13% separating and 5% stacking gels. After electrophoresis, gels were stained with Coomassie brilliant blue and the duplicate gels were electrotransferred onto nitrocellulose membranes and used in immunoblot experiments. In order to assess the different IgE-binding components possessed by the cultivars, sera from all fruit-sensitive atopic individuals with high SPT scores ($\geq 3+$) were pooled and used in immunoblots [1, 3]. In order to determine the major allergen components of the cultivars, pooled extracts from all cultivars were electrophoresed, electroblotted, cut into strips and used in immunoblots with 24 of the sera from the 32 SPT-positive patients. This was done so that an allergogram could be constructed. The detection method used here was modified using the "IgE bridge method". In this version, a rabbit anti-human IgE (Dako Immunoglobulins A/S, Denmark) second primary antibody was used as a bridge between human serum (the first primary antibody) and the enzyme-conjugated secondary antibody. This step was then followed after washing of membranes by the addition of affinity purified goat anti-rabbit IgG (Bio-



Fig. 1. The different cultivars in blocks of 1–18 as numbered on the photograph. 1 = Assela, 2 = Labana, 3 = Sabbaka, 4 = Rothana, 5 = Manifi, 6 = Naboot Ali, 7 = Barhi, 8 = Mabroom, 9 = Roshidia, 10 = Sokery, 11 = Khodri, 12 = Halaw, 13 = Seeleg, 14 = Juffair, 15 = Qattar, 16 = Shahala, 17 = Nabtat Jannah, 18 = Hagee. Cultivars will be referred to by their names in the text.

Rad Laboratories, Richmond, Calif., USA) conjugated to horseradish peroxidase (HRP). Enhanced chemiluminescence (ECL) (Amersham International plc, Bucks, UK) detection method was used according to manufacturer's guidelines followed by autoradiography. This method has increased sensitivity and made it possible to detect some of the weaker reacting bands [10]. The resultant autoradiographs were photographed and kept as a permanent record. The number of IgE immunostaining bands were counted for each cultivar and results recorded.

Results

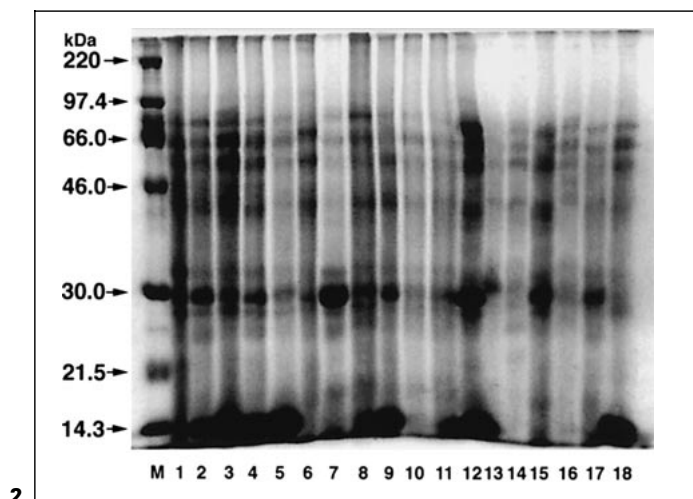
Cultivars were photographed for ease of identification as shown in figure 1 where distinct cultivar coloration, surface sculpturing, shape and sizes of fruits are seen. A comparison of the SDS-PAGE gel of the 18 extracts stained with Coomassie brilliant blue is shown in figure 2. The banding pattern of each cultivar shows that some bands differ in intensity from one cultivar to the other. The most common cluster of SDS-PAGE protein bands occurs at the molecular weight ranges ≤ 14.3 , 27–33, 36–39, 54–60 and at 60–70 kDa ranges in all cultivars. Sokery, Juffair and Shahala are the only cultivars that do

not have any particularly distinct bands. Seven of the cultivars namely Labana, Barhi, Mabroom, Roshidia, Halaw, Qattar and Nabtat Jannah possess peculiarly clearly-staining bands at 30 kDa.

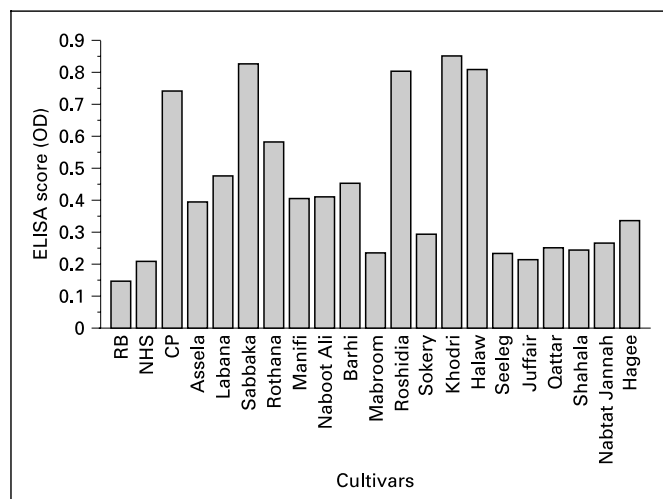
Since date fruits contain high proportions of sugar (ca. 60–70%) depending on the type of dates, and the involvement of carbohydrate epitopes in IgE binding is well documented, the protein/carbohydrate ratios were investigated. Results (not shown) indicated there were some differences between cultivars and this has formed the subject of further investigations.

The number of patients with SPT-positive reactions per cultivar is shown in figure 3. All cultivar extracts gave SPT-positive reactions in at least 1 patient with Sabbaka and Halaw being reactive in all patients. Eight of the cultivars were SPT-positive in $\geq 50\%$ (3–6) of patients. Manifi, Naboot Ali, Mabroom, Sokery, Juffair, Shahala and Nabtat Jannah were reactive in 2 patients each and Seeleg, Qattar and Hagee were only reactive in a patient each.

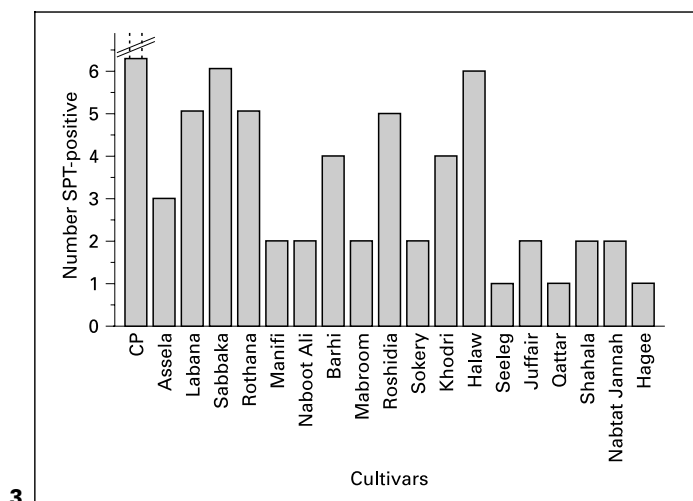
IgE ELISA results of cultivars with pooled atopic sera are presented in figure 4 and the results indicate that cultivars appear to give a gradation of ELISA scores. In this



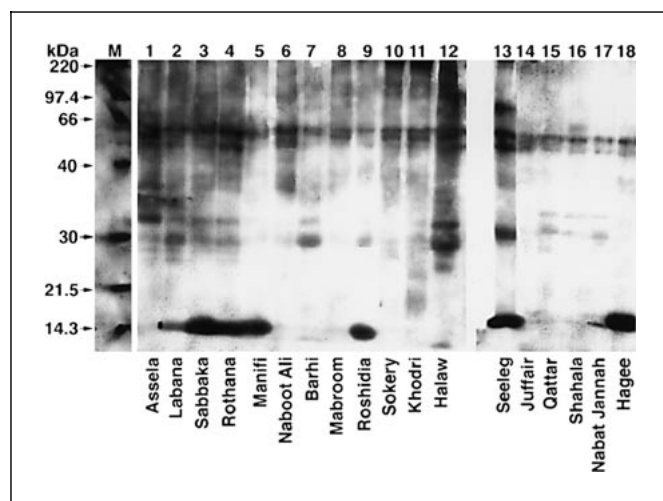
2



4



3



5

Fig. 2. Coomassie brilliant blue stained SDS-PAGE of Amersham rainbow molecular weight marker M (14.3–220 kDa M_r) and different cultivar extracts (lanes 1–18) as illustrated: 1 = Assela, 2 = Labana, 3 = Sabbaka, 4 = Rothana, 5 = Manifi, 6 = Naboot Ali, 7 = Barhi, 8 = Mabroom, 9 = Roshidia, 10 = Sokery, 11 = Khodri, 12 = Halaw, 13 = Seeleg, 14 = Juffair, 15 = Qattar, 16 = Shahala, 17 = Nabtat Jannah, 18 = Hagee.

Fig. 3. The number of patients that were SPT-positive per cultivar is represented as histograms. For the selection of allergenic cultivars, a cultivar was deemed allergenic if it elicited a positive response in ≥ 4 patients. CP represents the SPT response of patients to the extract pooled from all cultivars. Broken lines and slashes indicate that all the 32 patients initially skin tested were SPT-positive.

Fig. 4. IgE binding based on ELISA scores. A score of ≥ 0.56 was considered high enough for the cultivar to be selected for standardization. Lane 1 (RB) = reagent blank, lane 2 (NHS) = normal human serum pool, lane 3 (CP) = an extract pooled from all the cultivars and tested against the pooled atopic serum.

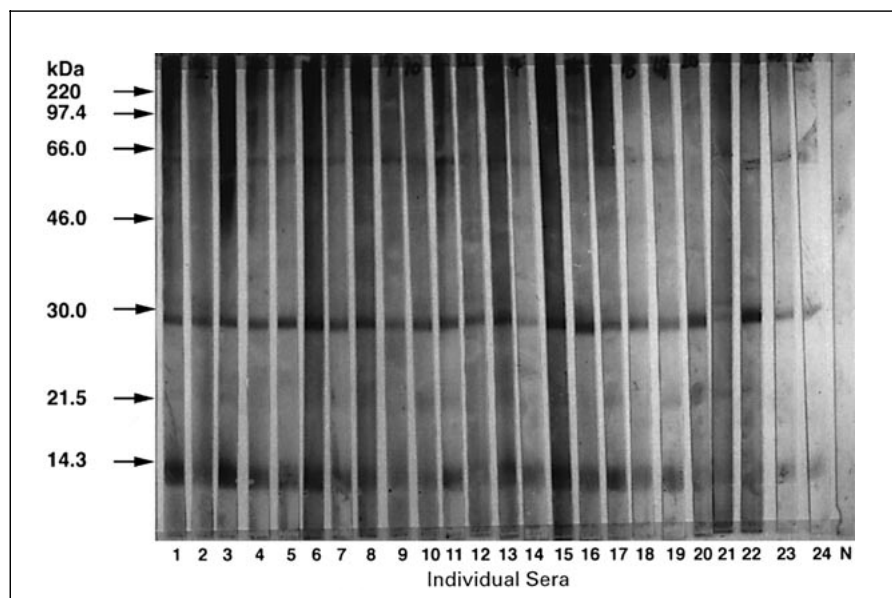
Fig. 5. IgE immunoblots of extracts with pooled positive sera: M = Amersham Molecular Weight Marker (kDa = kilodaltons as appended); numbers 1–18 are broken up because they were from two different SDS-PAGE gel transfers. Note that 13–18 is not to scale with lanes M and 1–12. The names of cultivars are as shown.

study, cultivars that attain 66% of the highest score of 0.84 (i.e. 0.56) are considered highly positive. Using this criterion, cultivars can be ranked as high IgE binding of ≥ 0.56 OD units (Sabbaka, Rothana, Roshidia, Khodri and Halaw), moderate IgE binding of 0.28–0.56 OD units (Labana, Rothana,) and low IgE binding for the remaining

cultivars that bind IgE below 0.28 OD units (Assela, Manifi, Mabroom, Sokery, Seeleg, Juffair, Qattar, Shahala, Nabtat Jannah and Hagee).

IgE immunoblot results of the individual cultivar extracts are presented in figure 5 and that of pooled extract with individual sera from 24 of the 32 atopic patients is

Fig. 6. IgE immunoblot of pooled cultivar extract and sera from 24 of the 32 patients who were SPT-positive to dates are presented in lanes 1–24. Lane N = pooled nonatopic normal human sera. In lanes 1–24 the main IgE binding is seen at 14.3 kDa and below, 27–33 and 54–58 kDa. The sera bind other bands to a lower extent at different molecular weights.



shown in figure 6. The number of IgE immunostaining bands as seen in the different cultivars varies from as few as 5 in Shahala, Nabtat Jannah and Hagee to as many as 12 in Halaw. What is striking about the bands in the different cultivars is that the bands at 54–58 kDa are present in all the 18 cultivars. The 30 kDa band is common to 16 out of 18 cultivars but this band varies in intensities between different cultivars. Consistent with the CBB stains of the bands, intensely staining globoid bands can be seen in some cultivars only. Halaw, Nabtat Jannah and Barhi are distinct in binding IgE to a very low molecular weight structure, which cannot be seen in other cultivars. All cultivars possess the 54–58 kDa cluster yet still it only binds IgE in 16 of the 24 atopic sera.

Considered together, these result indicate that there are 5 cultivars (Sabbaka, Rothana, Roshidia, Khodri and Halaw) which are consistent in their in vivo and in vitro allergenic characteristics, giving high SPT reactivities and high IgE-binding score as determined by ELISA. These same cultivars are also among the 8 that possess the highest number of IgE-reactive bands. It appears that Sabbaka and Rothana (lanes 3 and 4, fig. 5) bind similar IgE-binding structures while differences are apparent in CBB gel staining pattern (fig. 2).

To facilitate the selection of cultivars for standardization, an arbitrary score system was devised which took into consideration SPT reactivity and a number of IgE-binding properties (table 1). A score of 1 was assigned to a cultivar if it evoked an SPT reaction in 4–6 patients, had

an IgE ELISA score of ≥ 0.5 , bound IgE at ≤ 14.3 and 27–33 kDa, or possessed more than 8 IgE immunoblot bands. This criterion allowed each cultivar a maximum score of 5. Three cultivars (Sabbaka, Rothana and Roshidia) possessed all 5 of the selected properties and 4 cultivars (Labana, Barhi, Khodri and Halaw) possessed 4 of the properties. The foregoing indicates clearly that a lot of relevant allergenic properties were revealed by the use of this method of analysis.

Discussion

Dates are a staple food in the Middle East, North Africa, Egypt, Spain, parts of India and the hotter parts of the USA. In Saudi Arabia, the date palm is among the small number of plants that are indigenous and thrive in the harsh conditions of the dry arid environment. Due to the dioecious nature of the date palm, and the long period of cultivation in areas where it is used as a food crop, the palm is heterozygous and has resulted in the establishment of hundreds of varieties or cultivars. The allergenicity of date pollen has been established [10] and the allergenic variability of pollen from different cultivars of the date palm has been elucidated [3]. Since then, differences in the allergenicity of cultivars of other plants such as olives [11] pecan, wild chenopods and some amaranths have been demonstrated [12, 13].

Table 1. SPT and IgE-binding properties of cultivars

Cultivar		SPT- positive (4–6)	ELISA (OD ≥ 0.56)	IgE immunoblot binding			
				≥ 14.3 kDa	27–33 kDa	bands >8	total
1	Assela	0	0	0	1	1	2
2	Labana	1	0	1	1	1	4
3	Sabbaka	1	1	1	1	1	5
4	Rothana	1	1	1	1	1	5
5	Manifi	0	0	1	0	0	1
6	Naboot Ali	0	0	0	0	0	0
7	Barhi	1	0	1	1	1	4
8	Mabroom	0	0	0	0	0	0
9	Roshidia	1	1	1	1	1	5
10	Sokery	0	0	0	0	1	1
11	Khodri	1	1	0	1	1	4
12	Halaw	1	1	1	1	0	4
13	Seeleg	0	0	1	0	1	2
14	Juffair	0	0	0	0	1	1
15	Qattar	0	0	0	0	1	1
16	Shahala	0	0	1	0	1	2
17	Nabtat Jannah	0	0	1	0	0	1
18	Hagee	0	0	1	0	0	1
Pooled extract		1	1	N/A	N/A	N/A	N/A

High SPT positivity in 4 or more patients, high OD ELISA score of ≥ 0.56 , possession of a cultivar of ≥ 8 IgE immunoblot bands and the ability of a cultivar to bind IgE components of ≤ 14.3 and 27–33 kDa in immunoblots were used as properties in an allergenicity score table. Each property earned a cultivar ‘1’ (one) point and only cultivars scoring ≥ 4 were deemed suitable for inclusion in the preparation of an in-house extract and for allergen standardization.

Due to the importance attached to the ability of allergists to identify slight differences in allergens, which otherwise prevents identification and effective treatment, a matrix-structuring technique has been developed and successfully used for clustering of allergens in groups. As a result, allergens that were considered to be fully cross-reactive and indistinguishable can now be discriminated [14].

Food allergy or allergic reactions due to food additives has gained importance in recent years and cases of anaphylaxis to foods including dates have been reported [1, 2]. If susceptible individuals are able to identify the food or the particular variety or cultivar of the plant or animal food that they are allergic to, then it is easy for them to avoid those foods. In our setting, only a few date-allergic patients are aware of the specific date cultivars that they cannot tolerate. Due to the large number of date cultivars, regional differences in naming varieties and the inevitable taxonomic problems with unequivocal identification of certain cultivars, dates used in the present study were purchased from a local merchant. A photograph of the culti-

vars and their names (as labelled by the vendor) are shown for the ease of identification.

The methods used in this study include in vivo and in vitro IgE-based methods recommended for the preparation of in-house extracts and for allergen standardization [4, 15–17]. The recommended methods for defining composition of allergens are IgE-based methods such as crossed radioimmuno-electrophoresis or IgE immunoblotting. Other non-IgE-based methods that also define composition are SDS-PAGE, crossed immunoelectrophoresis and other protein and amino acid quantification methods [18]. We have used SDS-PAGE and IgE immunoblotting to define the composition of our cultivar allergens and also to identify the constituent major allergens.

Pooled extracts have been used in IgE immunoblots using sera of 24 atopic-reactive patients to construct an allergogram. This has helped to confirm the previous finding that the major allergens of the date fruits are peptides of ≤ 14.3 , 28–33 and 54–58 kDa. Additionally, in vivo SPT and in vitro IgE ELISA-based methods as recommended by the United States Food and Drug Administra-

tion and Nordic guidelines were employed to determine the potency of allergens [19]. It is also recommended that extraction of allergen source materials should be in such a way that it ensures high allergenicity and that all the important allergens are left in naturally occurring proportions. Furthermore, allergenic preparations for diagnosis should contain all relevant allergens or antigens present in proportions reflecting the composition of the source material. The Nordic guidelines that three different concentrations be tested in a large number of patients could not be satisfied due to the large number of cultivars tested [20, 21].

In this study the source of allergenic material, extraction methods, solutions and other procedures were consistent enough to ensure that all cultivar extracts were prepared in the same way and by the same operator. Temperature and pH were stringently controlled so that natural allergic constituents were unaltered or diminished.

Eight of these cultivars have been tested in a previous study using a larger number of patients and have been shown to exhibit differential reactivity in SPT. The specificity of pooled extract from these cultivars was also demonstrated in radioallergosorbent assay (RAST) with unabsorbed and preabsorbed (RAST inhibition) sera from fruit-sensitive individuals [1]. Results of SPT in the present study show a clear trend with 8/18 cultivars giving positive skin reactions in 50–100% of patients tested. Interestingly, 7 of these cultivars possess intense CBB staining SDS-PAGE bands at ≤ 14.3 and 30 kDa, except Khodri, which lacks an intense band at 30 kDa.

The number of IgE-reactive bands per cultivar reflect again that the highest number of immunostaining bands occur mainly in the same group of cultivars that exhibited high SPT reactivity. Sokery binds IgE to 8 bands but these bands occur in the 38 and 100 kDa range, probably minor allergens, and only shares a common band at 54–58 kDa with all other cultivars. This cultivar (Sokery) which exhibited low SPT reactivity (2/6) and low IgE binding in ELISA and can therefore be considered as of low allergenicity, is coincidentally one of the most popular cultivars preferred by most people throughout Saudi Arabia. IgE ELISA give a gradation of scores and would be a quick and useful method for selecting or screening for hyper- or hypoallergenic cultivars from the numerous cultivars that are grown countrywide. This method can also be used instead of skin testing date-allergic patients with numerous extracts as has been done in this study, ELISA can be used for quick screening and confirmatory diagnosis can then be done with SPT and other tests.

Skin prick test is the most popular method for initial diagnosis of allergic conditions and even though it is

usually difficult to correlate skin test results with in vitro IgE-based tests, results obtained in this study indicate that in the highly allergenic cultivars these parameters are closely correlated. Individual cultivar-specific IgE-binding patterns, as evidenced by the immunoblots, indicate that only certain cultivars bind IgE avidly at molecular weights of ≤ 14.3 and 27–33 kDa whilst all cultivars irrespectively, bind to the 54–58 kDa doublet. The relevance of this ubiquitous doublet should be thoroughly studied since a 65–67 kDa doublet of undefined reactivity was also found in date pollen. The only exceptions to this observation is that Barhi and Khodri do not bind IgE at ≤ 14.3 kDa even though this band is present in their SDS-PAGE CBB stains (lanes 7 and 11 in figure 2). It could well be that some components of those broad bands are not involved in IgE binding. They also possess, numerically, most of the IgE immunoblot bands and are also the same varieties that give high IgE ELISA score.

The final scoring system for selecting cultivars took into consideration all the properties that could confer allergenicity and this method was clearly able to discriminate between the cultivars in such a way that high allergenicity was as obvious as low or hypoallergenicity. It would have been an important addition to knowledge in this area of study if these in vivo and in vitro tests reported here were complemented with clinical studies addressing patients' tolerance or nontolerance to the different varieties. Due to the difficulty in patient recruitment, the number of cultivars/varieties to be used and the individuals to be tested in a well-controlled double-blind placebo, let alone ethical constraints pose a phenomenal task. More investigators and time are needed before this clinical aspect of the work can be fully investigated.

Our results indicated that cultivars which evoke high SPT reactivity, bind IgE in ELISA, possess the two main IgE bands (≤ 14.3 and 27–33 kDa) and possess >8 IgE immunoblot bands are the most allergenic of the cultivars. In our previous study [1] in which sera from date pollen- and date fruit-allergic patients were used in IgE immunoblotting, both groups of patients recognized common peptide bands of similar sizes in date fruit as have been shown in the highly allergenic cultivars here. However, in that study only about 50% of patients' sera bound IgE at 54–58 kDa. Studies on allergenicity of date palm pollen [3, 10, 12] and the present study on date fruits have all indicated that there are distinct differences in allergenicity and antigenicity of cultivars.

It can be concluded that the allergenicity of date fruit cultivars like in date pollen cultivars, is a cultivar-specific phenomenon. Date fruits possess three major allergens of

≤14.3, 27–33 and 54–58 kDa, and based on the findings here it is recommended that the 5 cultivars which are consistent in their in vivo and in vitro allergenic characteristics be selected for the preparation of 'in-house' allergen extracts and for allergen standardization.

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