

Allergens of *Borassus flabellifer* Pollen from Mumbai

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Abstract

Borassus flabellifer, the Palmyra palm, is the most common palm tree in Mumbai (India). The present study was undertaken to explore the *B. flabellifer* pollen allergy by skin prick test and nasal provocation test and also to identify major allergens by immunoblot analysis. The allergenic potential of the pollen extract was studied on 333 patients by skin prick tests (SPT) and nasal provocation test (NPT) with pollen extract of *B. flabellifer* on SPT positive patients. Skin prick testing on 333 respiratory allergic patients with whole pollen extract exhibited severe (4+) response in 22 (6.9%) patients. In 5/10 subjects, the NPT with allergenic extract of *B. flabellifer* caused definite decrease in nasal airflow. Allergen extract of *B. flabellifer* resolved in 28 protein bands in the molecular weight (MW) range of 10 to 100 kDa on 12.5% SDS - PAGE gel. Twelve protein bands with MW 20 to 100 KDa were allergenic and bound with specific IgE in the pooled patient's sera on immunoblotting. The IgE binding patterns of individual patients emphasize the predominance of 86, 76, 62, 45 and 40 kDa proteins in terms of allergenicity showing the major allergenic fractions in *B. flabellifer* pollen extract.

Key words: *Borassus flabellifer*, Pollen, Allergy, Nasal provocation test, Immunoblot

INTRODUCTION

The *Borassus flabellifer* (commonly known as Palmyra Palm) is native of tropical Africa. In India, the Palmyra palm is found in comparatively dry parts of Bihar, the coastal areas of West Bengal, Andhra Pradesh and Maharashtra states. In Mumbai city, the Palmyra palm trees are distributed mainly in western suburb and near the coastal areas. Various aerobiological studies from Mumbai city have stressed the abundance of *Barassus* pollen grains in the atmosphere^{1,2}. It has been reported to produce skin reactions in 31.64 % of the patients in West Bengal

with isolation of 90 - kDa component as one of the major allergen from *B. flabeliffer* pollen³. The present study has been carried out to evaluate the clinical and immunological role of *B. flabeliffer* pollen allergens in nasobronchial allergy patients from Mumbai.

MATERIAL AND METHODS

Pollen collection and preparation of extract:

Polliniferous materials were collected from male Palmyra palm trees at weekly intervals during the pollination season. The highly pure pollen (> 95%) sample defined as per the method of Cour and Loublier⁴, was defatted with diethyl ether. The antigens were extracted in 1:20 (w/v) Phosphate Buffer Saline (PBS, pH 7.5) by continuous stirring at 4°C for

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IJAAl, 2005, XIX (1) p 11-19.

24 hours on a magnetic stirrer. The extracts were clarified by centrifugation at 10,000 rpm for 30 min at 4°C. The supernatant was dialyzed against distilled water in visking dialysis tubing (with cut off point of 3500 daltons) for 24 hours. After dialysis, the extracts were centrifuged again at 10,000 rpm for 30 minutes at 4°C. The supernatant was then passed through Millipore filter (0.22µ). The extracts were lyophilized, stored at -20°C and reconstituted before experiments.

Soluble protein content in the whole pollen extracts was estimated by the method of Lowry et al⁵ using phosphotungstic acid (PTA) reagent to precipitate the proteins⁶ with bovine serum albumin as standard.

Subject selection:

A total of 333 individuals visiting three Asthma and Allergy Clinics situated in different parts of Mumbai were screened for allergic rhinitis and asthma. Thirty healthy individuals were also included in the study as negative controls.

Skin sensitivity test:

Skin testing was carried out on all 333 history positive (Asthma = 107, Rhinitis = 79, Urticaria = 21, Asthma + Rhinitis = 117, Asthma + Rhinitis + urticaria = 9) patients. Another thirty subjects were also subjected to skin testing as normal controls. The skin prick tests were performed on the volar region of the patient's forearms. Allergenic extract of *B. flabellifer* 1:10 w/v in phosphate buffer saline (PBS-glycerol) and the standard battery of antigen extracts were used for skin prick tests. Histamine (1mg / ml in 50% glycerinated PBS) served as positive control and glycerinated PBS as negative control. Skin reactions were graded after 20 minutes of the tests as per the criteria of Shivpuri and Agarwal, 1969⁷. Patients showing marked positive skin sensitivity (2+ and above) were selected for further clinical and immunochemical investigations.

Nasal provocation test:

Ten patients with marked positive SPT response were subjected to nasal provocation test⁸ with pollen extract of *B. flabellifer* under medical supervision. The response was evaluated by measurement of nasal airflow with active anterior, rhinomanometry, using

Connell Rhinometer (Jagger, Germany) and by scoring the subjective nasal and extra nasal symptoms of allergen sensitization. The clinical symptoms scored included rhinorrhea, nasal congestion, itching or burning of the nose, ear, palate, or throat; and sneezing and lacrimation (watery or red eyes). The intensity of these symptoms were expressed on the following scale: 0, none; 1, mild; 2, moderate; and 3, severe⁹. The control group consisted of 5 non-atopic and 5 atopic patients with a history of seasonal allergic rhinitis but having negative response to the SPT with pollen allergens under investigation. Subjects were made to rest for 30 minutes before the test to allow nasal mucosa to become acclimatized. At first, control solution (phosphate-buffered saline) was sprayed into each nostril with a metered dose pump delivering a fixed volume of 0.1 ml aqueous solution per puff. After provocation with control solution, 0.1 ml of increasing concentrations of *B. flabellifer* allergen extract (10, 50, 100 µg / 100 µl) applied in both nostrils at 10-minutes intervals. The standardization experiments revealed that the extract concentration of 100 µg / 100 µl is the optimum concentration required for testing. The Nasal provocation test (NPT) results were defined as positive, if nasal airflow decreased more than 40% in at least one nostril as compared with the post- PBS baseline value (the mean of 3 measurements following PBS administration was regarded as baseline value). This decrease in nasal airflow was sustained for at least two consecutive rhinomanometer measurements¹⁰ (i.e., it lasted at least for 10 minutes) and was accompanied by clinical symptoms persisting at least 30 minutes (the minimal score amounting to 3 or more). An increase in nasal resistance or a nasal blockage index was calculated by formula described elsewhere¹¹.

Statistical analysis: Data were analyzed using the chi-square test with Yates correction⁹.

Estimation of total and Specific IgE:

Total IgE in the patient's sera were estimated by the enzyme immunoassay using Quorum diagnostic EIA kit¹². Specific IgE to *B. flabellifer* were determined by ELISA as described by Vollar et. al.¹³. Briefly, lyophilized extract was reconstituted in carbonate-bicarbonate buffer (pH 9.6). One microgram per 100 µl was bound to each well of polystyrene

microtitre plate by overnight incubation at 4°C. After washing three times with PBST-20 (phosphate buffer saline with 0.05% Tween -20), plates were incubated with 3% bovine serum albumin (BSA) for 3 hours at room temperature. Plates were washed and incubated overnight at 4°C with 100µl of 1:10 (v/v) diluted individual patient's sera. As a negative control, normal human serum (NHS) from ten subjects with negative prick test to *B. flabellifer* pollen extract was also tested. After washing with PBST-20, bound IgE were detected by incubating the plate, overnight with 1:500 v/v HRP- conjugated anti-human IgE (ε-specific, Sigma, USA) at 4°C. After washing the plates, the color was developed by incubating with the substrate 0.5 mg / ml o-phenylenediamine, 0.006% hydrogen peroxide in 0.2 M potassium phosphate buffer, pH 7.0. The plate was incubated in dark for 30 minutes at room temperature. The enzyme reaction was terminated by adding 50µl of 2N HCl in each well and the absorbance was read using a ELISA reader (Dynatech, MR 5000). Patients sera showing a PR / NCR (Patients Reading / Normal Control Reading) ratio of 5 or more, were considered as ELISA positive.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS - PAGE) and Immunoblotting:

SDS-PAGE and Electrotransfer were carried out on 12.5% mini gel according to the buffer system

described by Laemmli¹². The protein bands resolved were visualized by staining with Coomassie brilliant blue solution. For western blotting, the SDS-PAGE separated proteins of *S. flabellifer* were transferred onto a nitrocellulose membrane as described by Towbin et al¹⁵. After the transfer, the unreacted sites on nitrocellulose membrane were blocked by incubation with 3% BSA in PBS for 3 hr at RT. After the blocking, the membrane was washed with PBS Tween-20 and cut into strips with single individual lane of separated pollen protein. These strips were incubated with (1: 10 v/v diluted in PBS, with 1% BSA) hypersensitive individual patient's sera (n=11) overnight at 4°C with gentle shaking. The nitrocellulose strips were washed with PBS- Tween-20 and incubated at 4°C with 1:500 v/v diluted peroxidase conjugated goat antihuman IgE (Sigma, USA). After washing the strips with PBST, the allergenic proteins were detected by treating them with a substrate solution (12 mg diaminobenzidine and 15 µl of 30% (v/v) H₂O₂ in 50 ml sodium acetate buffer, pH 5) for few minutes. The color reaction was, stopped by rinsing the strips in distilled water.

RESULTS

Pollen grains of *B. flabellifer* show ellipsoidal shape with 50-50 µm in diameter under scanning electron microscope (Fig 1). They are monosulcate and aperture

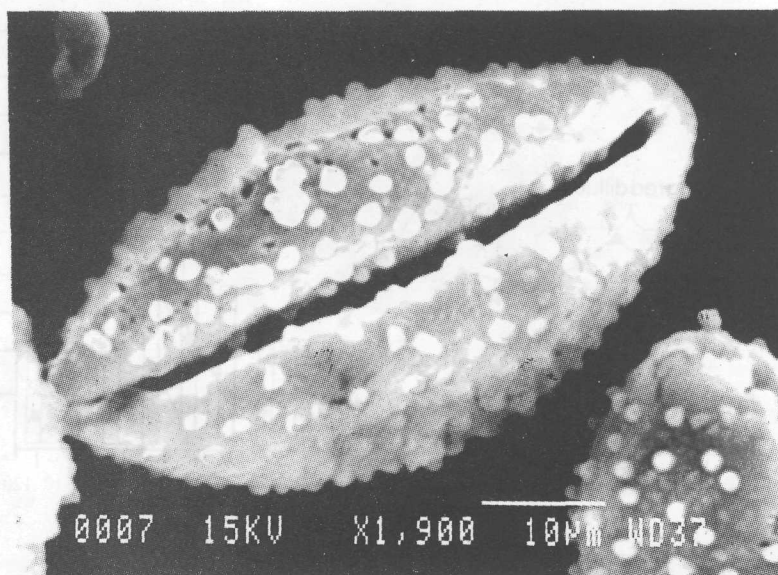


Fig. 1. *Borassus flabellifer* pollen under scanning electron microscope

extends from one pole to another on the distal side. Length of the aperture is approximately 45 - 50 μm and width of the aperture is approximately 3 - 4 μm . Exine has verrucate surface ornamentation. The Protein content of the pollen extract was found to be 0.0061 gm / ml (1:20 w/v. Skin prick test (SPT) with pollen extract elicited markedly positive skin reactions (4 +) in the 22/333 (6.9%). and 2 + to 3 + in 59/355 (17.7%) subjects.

Nasal Provocation Test (NPT)

In 5 of the 10, *B. flabellifer*-sensitive subjects, the nasal challenge test with pollen extract led to decrease in nasal airflow (fall from post-buffer diluent baseline value greater than 40%, lasting at least 10 minutes) during a 2-hour observation period. Fig. 2 shows positive NPT in one patient. In these 5 patients, the decrease was pronounced and accompanied by almost total nasal obstruction. Such fall in nasal airflow was frequently observed only in one nostril; A unilateral nasal block was noted in 3 patients (30%). After 100 μg / 100 μl antigen challenge, greater relative decrease in nasal airflow was observed within 15 minutes, in the subject with more subjective congestion. The correlation coefficient (r) was in the range of -0.11 to -0.93 in these 5 patients. Only in one patient (patient no. 4; aged, 43 years), significant decrease in nasal

airflow, and clinical symptoms were observed at the end of the second hour of the challenge. Increase of observation period for up to 3 hours led to moderate fall in nasal airflow accompanied by clinical symptom scores up to 4. The nasal blockage index was high (63.75%), thus the test results were positive in this patient.

In all the 5 patients with decrease of nasal airflow, watery discharge and the sensation of nasal blockage were noted during a 2-hour observation period. Other less frequent symptoms included itching of nose, ears, and/or palate (28%), sneezing (50%), and lacrimation (12%). The maximum intensity of the clinical symptoms ranged between a score of 3 to 10 points (average, points). Nasal symptoms appeared 10 to 70 minutes after challenge with pollen extract of *B. flabellifer* and lasted 30 to 120 minutes (average, 73.4 minutes). Nasal blockage index was higher (more than 60%) in 5 of the 10 (50%) patients indicating strong positive response to nasal challenge test (Table 1). The test results were mild positive in 2 / 10 (20%) control subjects without showing considerable decrease in nasal airflow and any concomitant nasal symptoms (Fig. 3). Nasal blockage index was in the range of 14% - 15.6% in 10 healthy subjects indicating negative response to nasal challenge test.

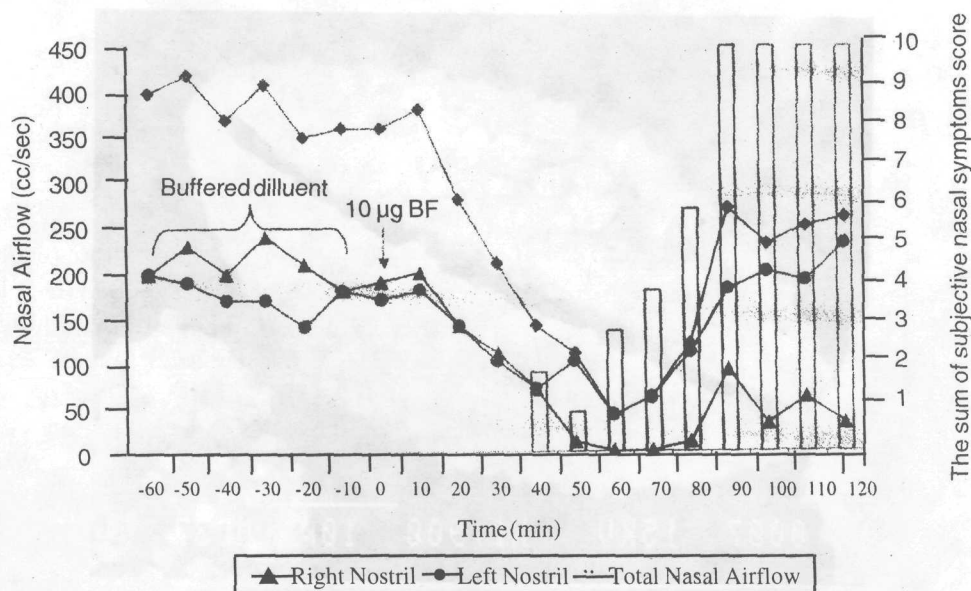
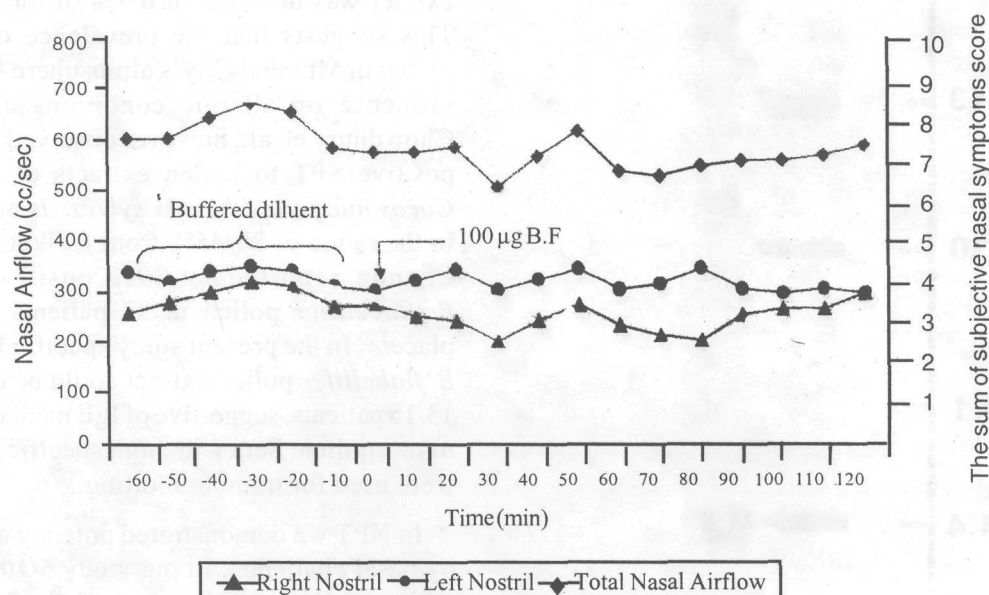


Fig. 2. NPT in patient with *Borassus flabellifer* allergy (Subject age, 49 years)

Table 1. Correlation between Nasal Blockage Index and average nasal Symptom score after challenge with *B. flabellifer* pollen extract

Nasal Blockage Index	N = 10 (%) score	Average Symptoms
Severe > 60%	5 (50)	2.86
Moderate 40 - 50%	-	-
Mild 30-40%	1 (10)	-
Controls 10-20%	9 (90)	-

**Fig. 3.** NPT in pollen extract of *Borassus flabellifer* (B.F.) in healthy subject (subject age, 42 years)

Total and Specific Serum IgE concentration

Total IgE values of >150 I.U. /ml were detected in 13/15 of *B. flabellifer* sensitive patients and in 9 atopic patients. *B. flabellifer* sensitive patients 11/15 showed a PR / NCR (Patients Reading / Normal Control Reading) ratio of 5 or more, indicating highly elevated specific IgE. Patients sera (n=4) showing total IgE values of > 500 IU / ml showed correspondingly high specific IgE PR / NCR ratio at 11.8, 12.1, 14 and 15.5 in *B. flabellifer* allergic patients (Table 2). Low specific IgE levels were observed in the normal control group.

SDS - PAGE and Immunoblotting

B. flabellifer pollen extract resolved into 28 protein bands on 12.5% SDS - PAGE gel in the MW range of 10 to 100 kDa after staining with Coomassie brilliant blue (Fig. 4) Out of 28 protein bands, 12 proteins of 20 to 100 kDa were detected on immunoblot with 11 (SPT and ELISA + ve) individual patient's sera. Amongst higher molecular weight proteins, maximum frequency of IgE binding was observed with 86, 76 and 62 kDa fractions.

Other high molecular weight proteins such as 100 kDa and 92 kDa showed lower IgE reactivity with

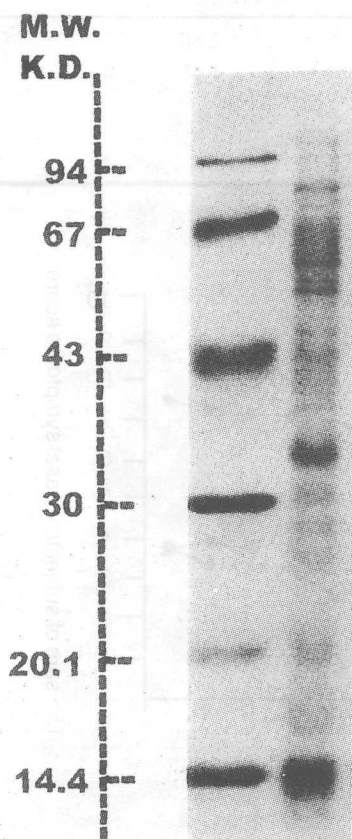


Fig. 4. Protein profile of *Boressus flabellifer* pollen extract on 12.5% gel. Lane 1: Molecular weight markers (SIGMA); 94 kDa - Phosphorylase b; 67 kDa Albumin; 43 kDa - ovalbumin; 30 kDa - Carbonic anhydrase, 20.1 kDa-Trypsin inhibitor and 14 kDa - α - Lactalbumin. Lane 2: *Borassus flabellifer* extract

54.5% and 45.4% patients' sera, respectively. Specific IgE antibodies from 72.7% of patient's serum samples recognized allergenic proteins of 70 and 55 kDa. Amongst lower molecular weight, allergenic protein bands of 31, 28 and 20 kDa were recognized by 45.4%, 54.5% and 54.5% of 11 individuals' patient's serum samples, respectively. Protein bands of 45 and 40 kDa were detected by specific IgE antibodies from 81.8% of patients sera tested. Protein bands of 86, 76,

62, 45 and 40 were recognized by more than 80% sera of patients, hence defined as major allergens.

DISCUSSION

B. flabellifer is a common palm tree growing wild or cultivated. Pollen grains are airborne almost throughout the year with pollen count 22 of contributing 2.28% to the total monthly catch¹ in the month of September. Skin sensitivity to its pollen extract was detected in 6.9% of the patients tested. This suggests that the prevalence of *B. flabellifer* pollen in Mumbai City's atmosphere has a significant influence on allergic conditions among patients. Chowdhury et. al., however observed the incidence of positive SPT to pollen extracts of *Areca catechu*, *Cocos nucifera*, *Phoenix sylvestris* and *B. flabelliter* in the range of 30-45% from Kolkata^{3,16}. Banik and Chanda reported 14.89% positive response to *B. flabelliter* pollen in 92 patients from the same place¹⁷. In the present study specific IgE reactivity to *B. flabellifer* pollen extract could be demonstrated in 13/15 patients, suggestive of IgE mediated sensitization to this pollen. Sera with high specific IgE titer (n=11) were used for immunoblotting.

In NPT we demonstrated potency of pollen extract by nasal challenge. In our study 5/10 (50%) patients with one dose application of *B. flabellifer* pollen extract on nasal mucosa demonstrated significant decrease in inspiratory nasal airflow and appearance of clinical symptoms such as watery discharge and nasal blockage. We continued to find a significant correlation between the extent of decrease in nasal airflow and the degree of subjective nasal congestion within 15 minutes, thus emphasizing the clinical relevance of quantitative rhinometric measurements. This could be due to the histamine released initially from mast cells as reported earlier by other workers¹⁸. In one patient, late phase reaction was noted at the end of the second hour of the challenge. Clinically, late phase response is noted by occurrence of nasal blockage, sneezing etc. at the end of 2- hours duration of the test and is considered to be derived primarily from early expression of the cytokines and chemokines, which leads to the recruitment and infiltration of inflammatory cells, reported by Pastorello et al¹⁹ and Zweiman et al²⁰.

Table 2. Total and specific IgE data for *Borassus flabellifer* sensitive patients

Number	Total IgE IU/ml.	Specific IgE O.D. at 495 nm	Specific IgE PR/NCR
1	400	0.486	72.00
2	281	0.610	91.00
3	810	0.942	14.05
4	435	0.523	7.80
5	785	0.812	12.10
6	124	0.161	2.40
7	259	0.362	5.40
8	325	0.256	3.82
9	290	0.310	4.62
10	100	0.292	4.35
11	480	1.041	15.50
12	150	0.869	12.90
13	414	0.792	11.80
14	530	1.031	15.20
15	345	1.212	18.08
Average	381.87	0.647	9.62
SD	±210.37	±0.334	±4.96
Range	100-810	0.161-1.212	2.4-18.08

P.R.: Patient's Reading,
N.C.R.: Normal control reading,

I.U.: International units.
Average N.C.R.: 0.067.

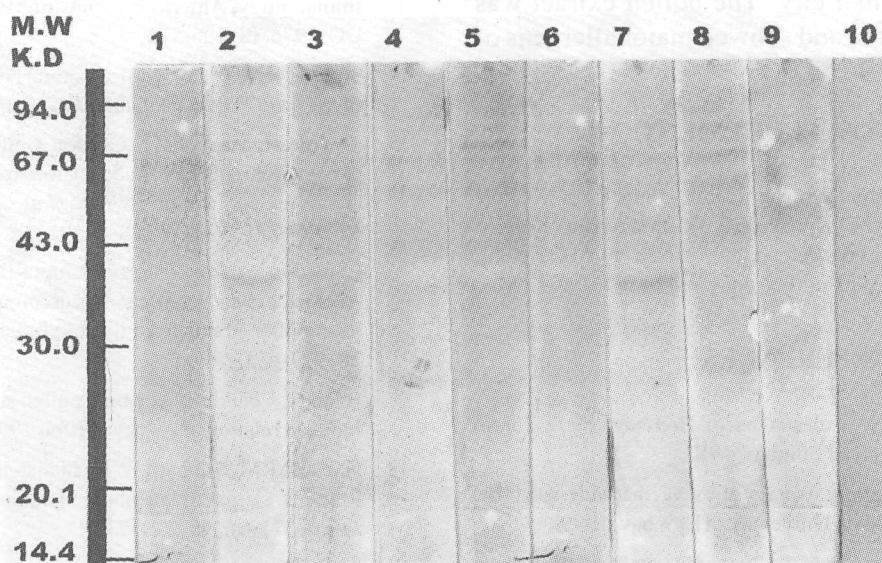


Fig. 5. Immunoblot showing IgE reactivity to *Borassus flabellifer* pollen extract with hypersensitive individual patient's sera (Strip No. 1 to 9) and pooled sera (Strip No. 10) of 5 control subject

In the present study SDS - PAGE resolved 28 fractions in *B. flabellifer* pollen extract with MWs 10 to 100 kDa. Immunoblot analysis showed 12 allergenic fractions in pollen with pooled patient's sera. The IgE binding patterns of individual patients emphasized the predominance of 86, 76, 62, 45 and 40 kDa proteins as major allergenic fraction in *B. flabellifer* pollen extract. The molecular weight distribution of IgE binding components, is quite similar to that observed in other studies. In an earlier study, allergenic extract of *B. flabellifer* resolved into 16-18 protein components, of which seven were IgE-reactive with positive pooled patients sera²¹. Further study has shown that the isolated 90-kDa component is one of the major allergens present in the pollen of *B. flabellifer*. Allergenic extract of *Areca catechu* pollen showed six IgE-reactive components between molecular weight range 18 to 116 kDa on immunoblot with pooled patient's sera. *Cocos nucifera* pollen extract resolved into 12-14 protein components on SDS - PAGE and five of them were IgE-reactive. *Phoenix sylvestris* pollen extract contained 14-17 protein components on SDS-PAGE whereas seven IgE-reactive components in immunoblotting²¹. Heterogeneity in IgE binding pattern of individual patients sera has been reported earlier by different workers^{22,23}. In conclusion *Borassus flabellifer* is an important source of pollen allergen for allergic individuals in Mumbai city. The pollen extract was partially characterized and showed major allergens of 86, 76, 62, 45 and 40 kDa.

ACKNOWLEDGEMENT

This research was supported by research grants from Medical research society of Sir H. N. Hospital and R. D. Birla Smarak Kosh of Bombay Hospital. The authors extend thanks to Dr. D. M. Tripathi, Hon. Allergologist for support and help in skin sensitivity tests.

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