

Comparison of antigenic and allergenic components of *Holoptelea integrifolia* pollen collected from different source materials

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Antigenic extracts prepared from pollen samples collected at weekly intervals during the same season did not exhibit significant variation in protein concentration. Stored pollen samples from different years, however, showed highly significant variations in protein concentration. The protein content of samples from different ecozones of India also varied ($CV = \pm 32\%$). The IEF and SDS-PAGE patterns were almost identical in samples from the same season, but were variable in the samples stored from different years and different parts of India. IgE binding proteins from different samples also varied depending on the overall protein profiles. Almost all the patients, however, showed IgE binding to four proteins at 50, 60, 66 and 70 kD, indicating the important allergenic components of *Holoptelea integrifolia*.

Key words: allergens; *Holoptelea integrifolia*; immunoblot; pollen antigens.

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Pollen of *Holoptelea integrifolia* is known to be an important tree allergen (1, 18, 20). The tree flowers for a short period of 2-3 weeks in February/March in various parts of India. The pollen concentration in the atmosphere reaches upto 41% of the total pollen concentration in urban areas (9, 11, 21, 24). In the standardization of allergenic extracts for diagnosis and immunotherapy, the investigation of pollen raw material prior to extract production is of paramount importance. Several factors are attributed to affect the quality of pollen, such as climate, method of collection, and storage conditions (2). Thus, antigenic extracts from the same species can vary in quality.

As *Holoptelea* is an important allergen and the tree is widely distributed and grows successfully in variable climatic conditions, we planned to investigate the antigenic and allergenic components of the pollen. During several flowering seasons the pollen was collected in the same

place and stored under conditions identical to those of pollen from the same season collected in different parts of India. The extracts prepared from different source materials were compared for antigenic and allergenic components using IEF, SDS-PAGE and immunoblot technique. The aim of the present investigation was to standardize the pollen raw material for preparation of allergenic extracts from *Holoptelea integrifolia*.

MATERIAL AND METHODS

Pollen collection

Polliniferous materials were collected from *Holoptelea integrifolia* at weekly intervals in March. After drying, pollen were procured by the natural shedding method and passed through different grades of sieves to remove other plant

material. Pollen collected during 1984 to 1987 and stored at 4°C were used to study the variations in pollen from different years. Pollen were also collected from Bangalore, Bhopal, Dehra Dun and Visakhapatnam, as well as from Delhi during the 1988 pollen season. The method of Cour et al. (3) was used to check pollen purity and samples were > 95 % pure.

Preparation of antigenic extracts

The antigenic components of pollen were extracted in phosphate buffer saline (pH 7.8) in 1:10 concentration by continuous stirring at 4°C for 20 h. The extracts were clarified by centrifugation at $27,000 \times g$ for 30 min. The supernatant was dialysed with visking dialysis tube (3,500 d cut-off point) against distilled water at 4°C for 24 h. Extracts were then centrifuged again, lyophilised and stored at -70°C till further use.

Determination of protein and protein nitrogen units

Protein concentration was determined by modified Lowry's method (13). Bovine serum albumin was used as standard. micro-Kjeldhal's method (8) was used for determination of protein nitrogen units (PNU). Proteins were first precipitated by phosphotungstic acid, precipitate was dissolved in 2 N NaOH and then used for PNU estimation. The mean protein and mean PNU concentrations and percentage coefficient variations were calculated to find the extent of variations among the samples studied.

Isoelectric focussing (IEF)

Ampholine PAGE plates (LKB-Pharmacia, Sweden, pH 3.5-9.5) were used for IEF. 100 µg of protein was loaded. pI markers obtained from Pharmacia (Sweden) were used to determine the pI of different proteins separated.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis

For SDS-PAGE, 10% gel containing 0.1% SDS was used in conjunction with Tris glycine

SDS buffer pH 8.4. 20 µl of samples, containing 80 µg of proteins were treated with 2 mercaptoethanol for 2 min at 100°C. Samples were then subjected to electrophoresis at 4 mA / well until the tracking dye reached 1 cm from the end of the gel. The gel was calibrated with marker proteins obtained from Sigma, (USA). The gel was stained with Coomassie Brilliant Blue R 250.

Serum samples

RAST positive sera from 12 patients from in and around Delhi were pooled to study the allergenic components of different extracts. However, sera from eight patients only were used to observe *Holoptelea* allergens by IgE binding to NC membrane. Sera were also collected from blood samples of five healthy non-atopic volunteers with low total IgE.

Electrophoretic transfer of proteins to nitrocellulose membranes

Proteins separated by SDS-PAGE were transferred to 0.45 µm NC for 5 h using Tris glycine buffer (25 mM glycine, 192 mM Tris, 20% methanol pH 8.3) at 30 mA at 4°C (23). After transfer, unreacted sites were blocked by 3% BSA in 0.3% TPBS for 3 h at room temperature.

Probing of NC membranes

After blocking of unreacted sites, strips were washed with PBS containing 0.5% Tween 20 (0.5% TPBS). Strips were incubated overnight with atopic sera (1:10) diluted with PBS containing 0.1% sodium azide. After extensive washing with 0.5% TPBS, strips were incubated with antihuman IgE peroxidase conjugate (Sigma) diluted to 1:500 with 0.3% TPBS for 3 h. After washing, colour development was performed with diaminobenzidine solution in dark (10 mg of 3-3 diaminobenzidine in 20 ml of PBS with 30 µl of hydrogen peroxide). Strips

Table 1.

Protein concentration in pollen stored from 1984-1989		
Years	Protein mg/ml	PNU/ml
1984	1.1	15,000
1985 *	-	-
1986	2.1	22,000
1987	1.1	11,200
1988 (1)	3.0	49,800
1988 (2)	3.9	55,600
1988 (3)	2.6	40,000
1989	3.2	50,000
Mean (CV %)	2.4 (41)	34,800 (49)

* Sample not available.

were stored in dark after treatment with 0.2 N HCl for 2 min and subsequent washing with distilled water.

RESULTS

Protein concentration

The protein concentration and protein nitrogen units of extracts prepared from pollen obtained from different years and places are given in Tables 1, 2. Protein and PNU concentrations of extracts prepared from the stored pollen varied from 1.1 mg/ml to 3.9 mg/ml (mean $2.4 \pm 41\%$ CV) and 11,200 to 55,600 (mean $34,800 \pm 49\%$ CV) respectively. However, when three samples from the 1988 season were analysed separately, variation was not significant ($CV \pm 17\%$ and $\pm 13\%$). The antigenic extracts prepared from

Table 2.

Protein concentration of pollen collected from different eco-climatic zones of India during 1988 season		
Places	Protein mg/ml	PNU/ml
Bangalore	1.6	20,000
Bhopal	3.5	53,400
Visakhapatnam	1.9	22,000
Dehra Dun	3.2	50,000
Delhi	3.9	55,600
Mean (CV %)	2.8 (32)	40,200 (40)

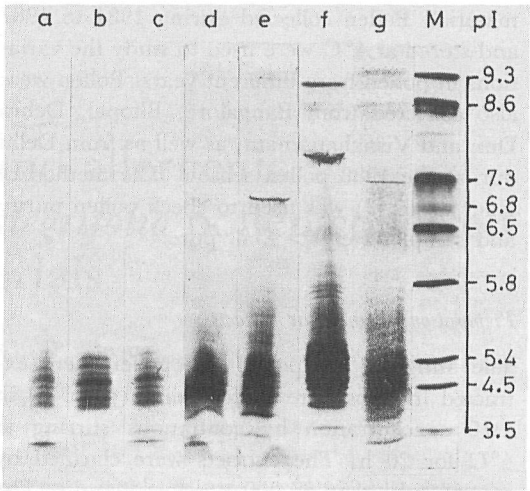


Fig. 1. IEF patterns of seven samples of *Holoptelea integrifolia* collected from Delhi and stored. a) 1984; b) 1986; c) 1987; d, e, f) 1988 (collected at weekly intervals) and g) 1989. M = Marker proteins.

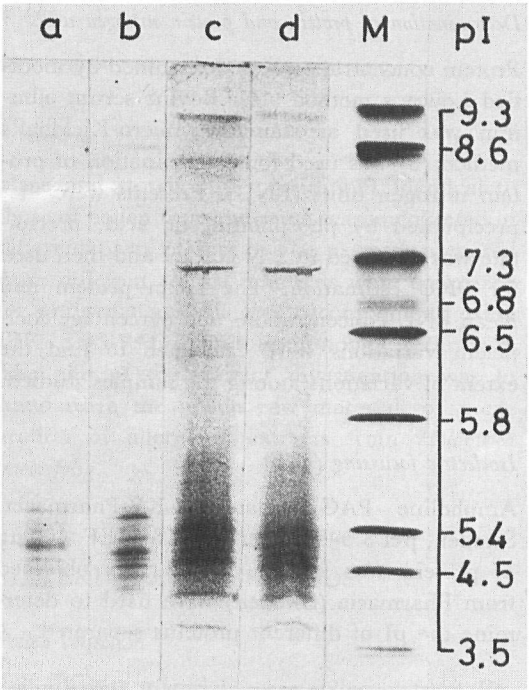


Fig. 2. IEF patterns of four samples of *Holoptelea integrifolia* from different ecozones of India collected during 1988 pollen season. a) Bangalore; b) Bhopal; c) Dehra Dun; d) Visakhapatnam. M = Marker protein.

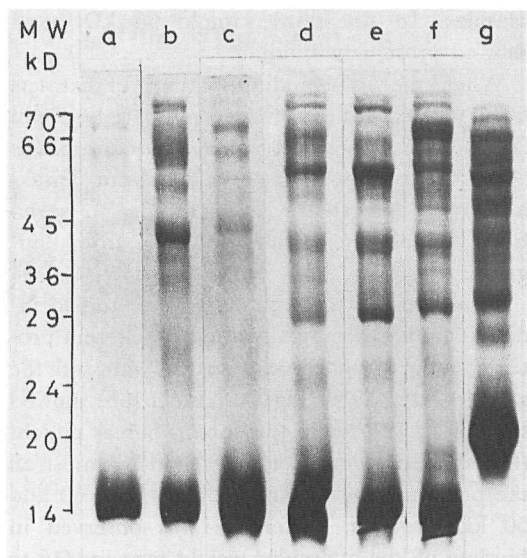


Fig. 3. Patterns of SDS-PAGE from seven samples of *Holoptelea integrifolia* collected from Delhi and stored. a) 1984; b) 1986; c) 1987; d, e, f) 1988 (collected at weekly intervals) g) 1989.

pollen collected from different areas (Table 2) varied from 1.6 to 3.9 mg/ml (mean $2.8 \pm 32\%$ CV) and PNU from 20,000 to 55,600 (mean $40,200 \pm 40\%$ CV).

IEF patterns

IEF patterns of the different extracts including three samples from 1988 are given in Fig. 1. The number of bands in pI 3.5–9.5 varied from 12 to 22 in the samples with maximum from (1f) and minimum from (1a). Around 10 bands are present in the pI range of 3.5–5.5 in most of the samples with distinct differences in staining intensities. The samples from 1988 and 1989 had a higher number of bands compared with samples from other years. Of the three samples (1 d, e, f) collected at weekly intervals during the same season (1988), bands in the alkaline region were more prominent in the third (1f) sample. When the IEF pattern of samples from different areas (Fig. 2) were compared, bands in the alkaline region were present in the samples from Delhi (1f), Dehra Dun (2c) and Visak-

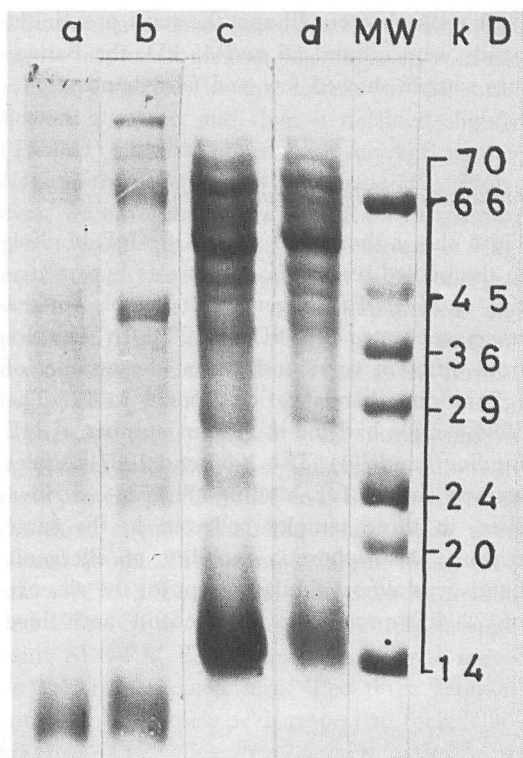


Fig. 4. SDS-PAGE patterns of four samples of *Holoptelea integrifolia* collected during 1988 pollen season from different areas in India. a) Bangalore; b) Bhopal; c) Dehra Dun; d) Visakhapatnam. MW = Marker proteins.

hapatnam (2d). In samples from Bangalore and Bhopal weak bands were observed in acidic/alkaline regions (Fig. 2a, b).

SDS-PAGE patterns

The heterogeneity in the patterns of pollen extracts obtained in SDS-PAGE are shown in Figs. 3 and 4. Samples from 1988 and 1989 (Fig. 3d, e, f, g) showed maximum number of visible bands, while those from 1984, 1986 and 1987 (3a, b, c) ranged from 14 kD to 70 kD. The banding pattern of three samples collected at weekly intervals was almost similar except for a prominent band of 66 kD in the third sample (Fig. 3f). When samples from different areas were compared, Visakhapatnam and Dehra Dun samples showed similar banding pattern.

In the sample from Bhopal the most prominent bands were around 66 and 45 kD, the Bangalore sample showed few and faint bands.

Identification of IgE binding proteins

Fig. 5 shows the results of specific IgE binding to the pooled sera from 12 patients hypersensitive to *Hološtelea* antigen, with the blot of extracts separated by SDS-PAGE. In samples from different years and places, the number of IgE binding bands varied from 4 to 12. The 1984 sample had the minimum number of IgE binding bands (4). The 1986 and 1987 samples showed similar IgE binding (Fig. 5b, c). However, in three samples collected in the same season, electrophoretic mobility of allergenic bands was almost similar except for the absence of 22 kD protein in the second and third

samples. In the third sample 66 kD band showed prominent binding.

When the IgE binding pattern of extracts from different areas were compared, Delhi (Fig. 5e, f) showed maximum allergenic bands (12) compared with nine bands from Visakhapatnam and Dehra Dun (Fig. 5i, j), seven from Bhopal (Fig. 5g) and only four from Bangalore (Fig. 5h).

When sera of eight patients were studied individually for their IgE binding to different proteins against pollen extracts of one of the samples from 1988 (Fig. 6), patient (6b) showed binding to 12 protein fractions, whereas patient (6h) showed seven allergenic bands. Almost all the patients showed binding to 70, 66, 60 and 50 kD proteins. Difference was observed in binding of low molecular weight proteins (16 to 36 kD). The pooled sera from healthy volunteers showed faint binding to 66 kD protein.

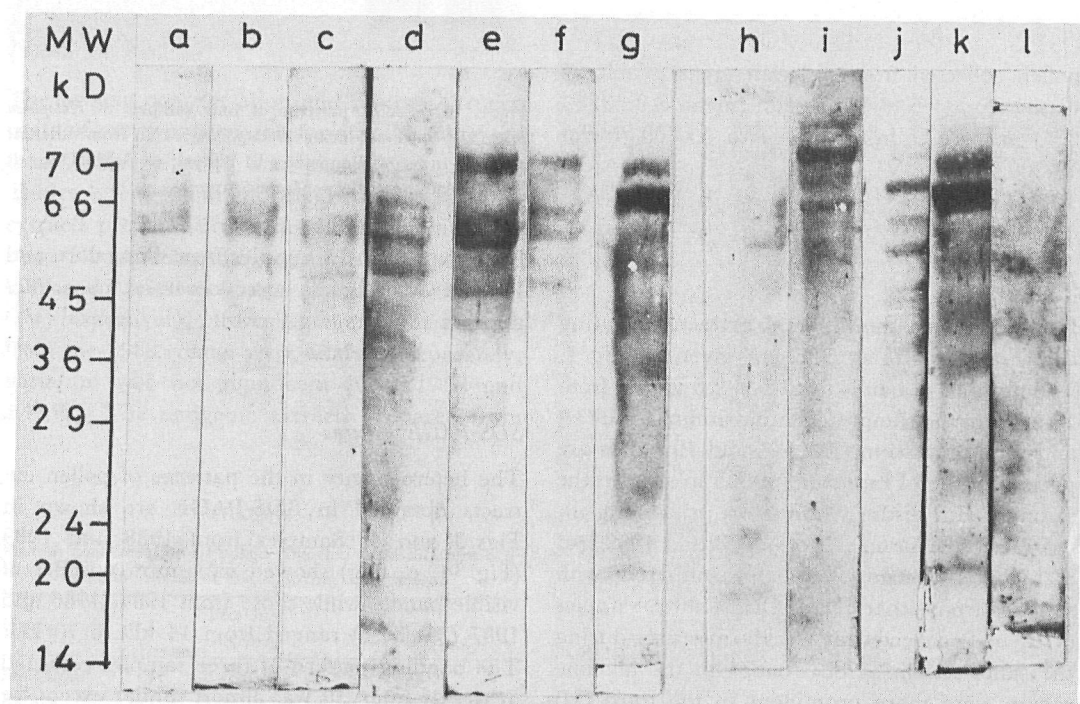


Fig. 5. Identifications of IgE binding proteins transferred to NC membranes after SDS-PAGE of *H. integrifolia* pollen extracts from different years and places in India. NC strips were incubated with pooled sera from 12 allergic patients. a) 1984; b) 1986; c) 1987; d, e, f) 1988; g) 1989; h) Bangalore; i) Bhopal; j) Dehra Dun; k) Visakhapatnam; l) control (incubated without any serum).

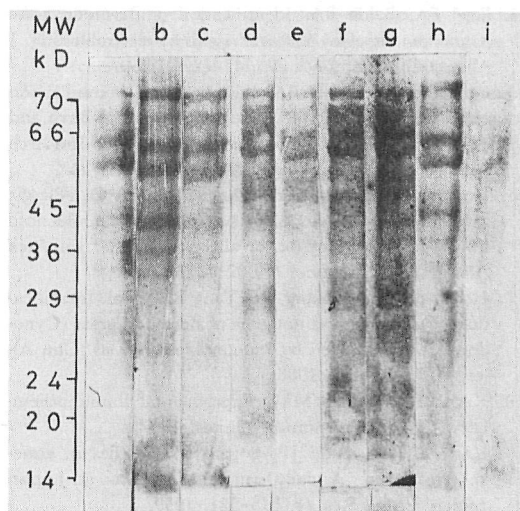


Fig. 6. Immunoblot of SDS-PAGE from pollen of *H. integrifolia* comparing the IgE binding proteins in eight highly sensitive patients. a-h) sera from allergic subjects; i) sera from non-atopic healthy subjects.

When no patient serum was used there was no band.

DISCUSSION

Since the quality of pollen determines the quality of the final antigenic extract used for diagnosis and immunotherapy, it is essential to have detailed information about pollen of the same species procured from different sources. Many workers have studied the antigenic extracts from different manufacturers and from different years and places. Both variations and similarities have been observed with these (2, 5, 6, 15, 16). Since the pollen present in the air in tropical countries vary more than those in temperate climates, we investigated three samples of *Holoptelea* pollen collected at weekly intervals during the same season and stored from 1–5 years, and also from five different ecozones of India with variable climatic conditions.

When all samples from different years were compared, protein concentration showed more than three-fold variation ($CV = \pm 41\%$ and $\pm 49\%$). However, samples collected in the same season from Delhi showed no significant

variation in protein concentration. A slight increase in protein content was observed with the sample collected in the middle of the brief flowering season and then it declined slightly (Table 1). Similar observation for AgE in *Ambrosia* anther extract was also made (12). However, we have observed a significant increase in protein concentration in pollen extracts of *Ricinus communis*, another important pollen allergen which has a fairly long pollination period (unpublished). But no decrease in protein concentration was seen towards the end of the flowering period.

Samples from different years were not in agreement and showed significant variations in their protein profiles. IEF and SDS patterns of the three samples from the same season were almost similar. The samples from 1984, 1986 and 1987 had fewer bands than those from 1988 and 1989. Of the 20 bands seen in the samples using SDS-PAGE, 12 showed binding to specific IgE from pooled sera. The three samples corresponded closely with respect to their allergenicity. The pollen used in the current study had more allergenic bands than stored pollen. This suggests that stored pollen loses its allergenicity because of such factors as moisture content, climatic conditions and method of collection (2, 15).

Pollen samples collected from five different ecozones of India, during the same pollen season (1988) showed significant variation in protein content (Table 2). Variations were also seen in the samples when subjected to IEF and SDS-PAGE. Visakhapatnam and Dehra Dun samples had similar and more bands than Bangalore and Bhopal, respectively. Samples from Delhi, Dehra Dun and Visakhapatnam had similar allergenic profiles, whereas Bangalore and Bhopal showed poor allergenic epitopes. Variation in the IgE binding patterns could be due to patients not being sensitized to the specific proteins in these samples. The variations could also be due to differences in climate and soil, time of collection, and maturity of inflorescence even during the same pollination season.

Although batch-to-batch and seasonal differences and similarities have been shown for *Am-*

brodia, *Phleum pratense* and *Lolium perenne* pollen (2, 14–16, 22) there is little information available about important pollen allergens of India. Most studies have shown the binding pattern of individual sera to pollen-sensitive patients to be heterogeneous (4, 7, 17). However, the IgE binding pattern of sera from eight patients against fresh pollen antigen used for SDS-PAGE corresponded closely. All patients' sera bound to 70 kD, 66 kD, 60 kD and 50 kD proteins, thus demonstrating these to be the most important allergenic components. No patient showed antibodies to all components of antigenic extracts, thereby suggesting that none of them was sensitized against all proteins. In pooled sera from healthy controls, IgE binding was seen against 66 kD protein; however, the intensity of binding was poor. This could be due to some controls being atopic to cross-reacting antigens.

Conclusions

Our investigations suggest that for mass preparation of allergenic extracts, the time and place of pollen collection, together with the period of storage, should be assessed on a pilot scale, before processing, to achieve uniform results. As far as possible fresh pollen should be used. The IEF, SDS-PAGE and immunoblot techniques provide ample indication for appropriate raw materials to be processed.

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