

Clinico-Immunologic Evaluation of Allergy to Himalayan Tree Pollen in Atopic Subjects in India - A New Record

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SUMMARY Exposure to local pollen allergens has a direct bearing on the prevalence of allergic symptoms among the inhabiting atopic population. The populations in the Himalayas and around it are exposed to a variety of pollen grains from trees growing in the region, but the pollen-population interaction has not been clinically investigated. Himalayan tree pollen from five different taxa, i.e. *Alnus nitida* (AN), *Betula utilis* (BU), *Cedrus deodara* (CD), *Mallotus philippensis* (MP) and *Quercus incana* (QI) were evaluated for their allergenicity in the Indian population by *in vivo* (skin prick test) and *in vitro* (ELISA) clinico-immunological methods. The presence of specific IgE against these tree pollen in the sera of skin test positive patients was taken as evidence for sensitization to these pollen. The average skin positivity in atopic populations recorded at different allergy centers in India varied from 2.2% against AN, to 4.7% against MP pollen. Significantly raised specific IgE against these pollen were observed in the sera of hypersensitive patients. The sensitization pattern to Himalayan tree pollen in these atopic populations varied. It was concluded that skin prick test positivity and raised IgE antibodies specific to AN, BU, CD, MP and QI established Himalayan tree pollen as important sensitizers in the atopic populations of India. A high incidence of skin sensitivity was observed to pollen antigens of *Cedrus deodara*, *Mallotus philippensis* and *Quercus incana* in patients of Chandigarh residing in the hills and foothills of the Himalayas while *Alnus nitida*, *Betula utilis* and *Cedrus deodara* were important sensitizers in Delhi patients. The skin sensitization pattern against these pollen was in accordance with the level of exposure to these pollen of the subjects residing in that part of the country.

Among airborne bioparticulates, pollen grains are important precipitating factors for allergic symptoms such as allergic rhinitis, bronchial asthma and atopic dermatitis.¹⁻⁴ However, allergic pollens are variable in different ecozones of the world, as the vegetation of one region differs markedly from another. Pollen of ragweed, plantain, olive, oak, alder, birch and *Acacia* are reported to be allergenic in the USA.^{5,6} In Europe, pollen of ragweed, birch, *Parietaria judaica* and grasses are considered important allergens,³ but in Japan the Japanese Cedar (*Cryptomeria japonica*) is the major cause of pollinosis.⁷ Similarly in Korea the Japanese hop is reported to be highly allergenic.⁸

A sizable population inhabiting the Himalayan range suffers from allergic disorders. Several allergenically important taxa were identified from different ecogeographical regions of the country based on clinical and immunological evaluations,^{4,9} but information on allergy causing-pollen of the Himalayan region has been scarce except sensitization to *Cedrus deodara* reported by us earlier.¹⁰ Hence it

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was important to identify additional causative aeroallergens from every region of India for an effective diagnosis and treatment of patients with respiratory allergy and an improved practice of allergology in this subcontinent.

The pollen of alder- *Alnus nitida* (AN), birch- *Betula utilis* (BU), deodar- *Cedrus deodara* (CD), kamla- *Mallotus phillipensis* (MP) and oak- *Quercus incana* (QI) were reported to be in the air in sufficient quantity to sensitize an exposed population during their pollen seasons.⁹⁻¹³ These pollen were also reported to have drifted more than 300 km away from the Himalayas exposing additional populations.⁹ In the present work we have investigated a panel of allergenically important tree pollen from the Himalayas for the first time by skin prick test and allergen specific IgE antibodies in the sera of allergic subjects in different centers in India.

METHODS

Biology of the selected Himalayan trees

The habitat, geographical distribution and flowering season of the trees selected for this investigation are provided in Table 1.

Collection of pollens

The polliniferous materials from AN, BU, CD, MP and QI were collected during their respective pollen seasons from the Shivalik range of the Himalayas (Table 1). The samples were microscopically examined for purity as per the method outlined by Cour and Loublier.¹⁴ The percentage purity was calculated based on more than 1,000 particles of the studied pollen, plant parts, other pollens/fungi and

dust particles if any. Samples having a pollen purity of > 95% were used for antigen extraction.

Extraction of pollen antigens

Extraction was carried out in phosphate buffered saline (pH 7.8) 1:20 (w/v), at 4°C by continuous stirring for 20 hours. After centrifugation, antigens were dialyzed, lyophilized, and stored at -70°C for further experimentation.

Selection of respiratory allergy patients for skin testing

Patients with a history of respiratory allergy in the age group of 10 to 50 years, visiting the V.P. Chest Institute, Delhi, the Postgraduate Institute of Medical Education and Research, Chandigarh, the Institute of Child Health, Kolkata, or the Medical College, Thiruvananthapuram, were selected for skin prick testing. Patients aged 10-16 years were combined in a children group, while the rest were considered adults. Chandigarh is situated at the foothills of the Western Himalayas, 200 km from Delhi, which is also influenced by the Himalayan climate. The other centers were far away from the Himalayas and were selected as control centers to compare sensitization patterns.

A detailed medical history of the patients was recorded by the physicians and the patients were examined by routine tests such as chest X-rays, blood, urine and pulmonary function tests to rule out any other disorder.

Skin prick testing

Subjects diagnosed as patients with naso-

Table 1 Geographical distribution of Himalayan trees studied for their allergenicity

Tree Pollen	Geographical distribution	Elevation (meters)	Flowering period
<i>Alnus nitida</i>	Western Himalayas	900 – 2,700	Sep-Dec
<i>Betula utilis</i>	Inner Himalayas	3,000 – 4,200	Mar-Apr
<i>Cedrus deodara</i>	Western Himalayas	1,300 – 4,000	Aug-Dec
<i>Mallotus phillipensis</i>	Throughout India/ Western Himalayas	up to 1,500	Oct-Dec
<i>Quercus incana</i>	Western Himalayas	1,000 – 2,400	Aug-Oct

bronchial allergy were included for skin testing with their informed consent. Not all pollen antigens were tested on all patients at the different allergy centers. Skin prick tests with 1:10 (w/v) were performed on the volar surface of the forearm with 23G sterile disposable needles. Histamine hydrochloride (1 mg/ml) and 50% glycerinated buffered saline were used as positive and negative controls, respectively. Skin tests were graded as per the criteria of Singh *et al.*¹⁵ Patients showing wheals of 3 mm (1+) or more were considered positive. Patients showing 2+ and above skin reactivity were considered markedly positive. Alongside the Himalayan tree pollen other routine pollen and fungal allergens were also tested on those patients (Table 2).

Blood collection

Venous blood (10 ml) was collected from the test subjects, and sera were separated and stored at -70°C for immunoassay studies (ELISA and immunoblotting).

Enzyme-linked immunosorbent assay (ELISA)

Allergen specific IgE antibodies were measured by indirect ELISA according to the method outlined by Sepulveda *et al.*¹⁶ and followed by us in our earlier study.¹⁰ Briefly, polystyrene microtiter plates (Nunc, A/S Roskilde, Denmark) were coated with 100 µl of pollen extracts (200 µg/ml protein) and incubated overnight at 4°C. The free sites were

blocked with 1% BSA. After incubation 100 µl of sera, diluted 1:10 (0.05% BSA in PBS), were added to each well and incubated at 4°C. Anti-human IgE antibodies labeled with alkaline phosphatase (diluted 1:1,000) were added to each well and incubated at 37°C for 4 hours. For color development *p*-nitrophenyl phosphate was added, the reaction was terminated after 45 minutes and O.D. values were taken at 410 nm.

Analysis of ELISA results

Analysis of the OD values of the serum samples was done according to the criteria of Kauffman *et al.*¹⁵ with slight modifications. The binding percentage was calculated for each sample relative to the control sera as well as a pool of highly positive sera. Depending on the binding percentage, optical density (OD) values were categorized into 2 groups, i.e. with < 15% and > 15% binding. However, sera showing more than 30% binding were considered to have significantly raised ($p < 0.05$) specific IgE against respective pollen allergens. The sensitivity and specificity of the ELISA test as compared to the skin prick test results were calculated as described by Blay *et al.*¹⁶

RESULTS

Demography of the patients tested

The total number of patients tested at the different centers with different pollen extracts varied from 141 patients for AN to 531 to CD (Fig. 1) depending on the suitability of the patients available at the different medical units. The male/female ratio of the patients tested was approximately 2:1 against AN and CD extracts, respectively. The number of males tested against each extract was higher than the number of females. The number of children tested was lower than that of adults. Most of the atopic patients were in the age group of 20-40 years.

Symptomatology of patients tested

Patients included in the study at different medical centers were diagnosed in three groups based on the nature of their symptoms, i.e. allergic rhinitis, allergic rhinitis with asthma, and only asthma (Fig. 1). About 50% of the patients tested

Table 2 List of common inhalant allergens tested along with Himalayan pollen allergens on atopic patients at different centers

Pollen allergens	Fungi/dust mites
<i>Amaranthus spinosus</i>	<i>Alternaria alternata</i>
<i>Artemisia scoparis</i>	<i>Cladosporium herbarum</i>
<i>Chenopodium album</i>	<i>Aspergillus fumigatus</i>
<i>Holoptelia integrifolia</i>	<i>Aspergillus versicolor</i>
<i>Prosopis juliflora</i>	<i>Rhizopus nigricans</i>
<i>Morus alba</i>	<i>Epicoccum nigrum</i>
<i>Cynodon dactylon</i>	<i>Curvularia lunata</i>
<i>Genchrus ciliaris</i>	<i>Dermatophagoides farinae</i>

suffered from both, allergic rhinitis and bronchial asthma. Their percentage varied from 48.8% of those tested against CD to 52.9% against MP. However, 22.7% patients tested against AN and 29.8% against QI had symptoms of bronchial asthma only, while 21.5% of those tested for allergenicity to CD, and 29.1% of those tested against AN were suffering from allergic rhinitis alone. Seasonal allergic cases varied from 39.8% to 43.5% for AN and QI, respectively. Seventeen and a half percent of those tested against CD and 29.1% for AN were perennial cases. However, irregular symptoms throughout the year

were recorded in 33.4% of patients tested with MP, while 40.8% patients tested with CD reported irregular symptoms.

Skin test results

The results of the skin prick tests conducted with tree pollen antigens of AN, BU, CD, MP and QI on respiratory allergy patients attending medical clinics at four different centers in India are provided in Table 3.

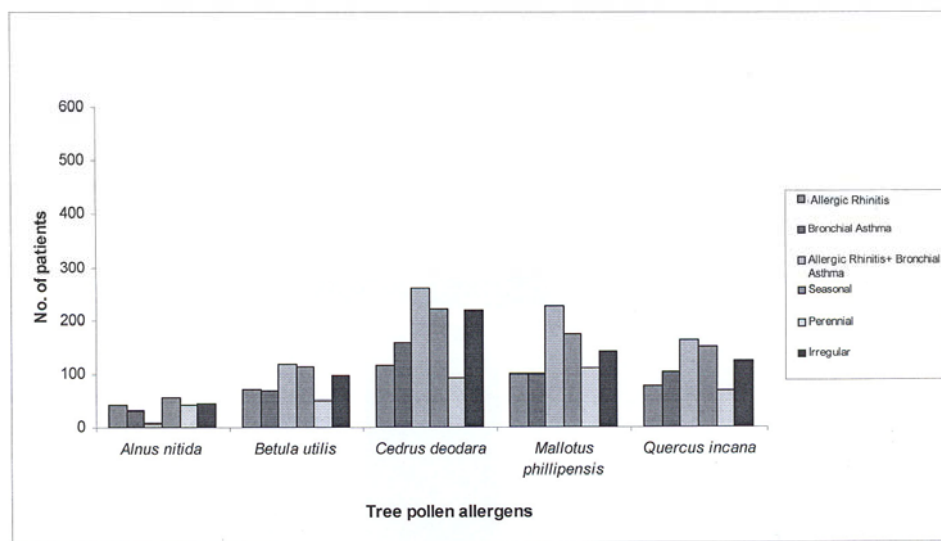


Fig. 1 Symptoms and seasonality of the patients selected for skin prick testing against Himalayan tree pollen extracts

Table 3 Results of the skin prick test with tree pollen extracts of patients with respiratory disorders at four different allergy centers in India

Antigens	Chandigarh			Delhi			Kolkata			Thiruvananthapuram		
	Total	Skin positivity		Total	Skin positivity		Total	Skin positivity		Total	Skin positivity	
		1+ to 3+ %	2+ to 3+ %		1+ to 3+ %	2+ to 3+ %		1+ to 3+ %	2+ to 3+ %		1+ to 3+ %	2+ to 3+ %
<i>Alnus nitida</i>	84	5.9	1.2	57	14	3.5	*	*	*	*	*	*
<i>Betula utilis</i>	*	*	*	155	9	6.4	104	0.96	0.96	*	*	*
<i>Cedrus deodara</i>	86	12.7	9.3	182	9.3	7.1	200	27	8.0	63	12.6	4.7
<i>Mallotus phillipensis</i>	84	13.1	4.8	60	5	1.6	218	32.5	7.3	63	25.3	4.7
<i>Quercus incana</i>	84	15.5	8.3	258	4.2	3.4	*	*	*	*	*	*

*Not included for testing

Pollen extracts of AN, CD, MP and QI tested at Chandigarh showed maximum skin positivity (2+ to 3+) to CD extract (9.3%), followed by 8.3% against QI and 4.8% and 1.2% against MP and AN pollen extracts, respectively.

At Delhi overall positivity to AN was 14% but with marked positivity (2+ and above) in 3.5% patients only. Pollen of CD showed 9.3% positivity at Delhi with 7.1% of marked positivity.

At Kolkata, a high sensitivity was observed in 27% and 32.5% cases to CD and MP pollen, respectively, but 2+ and above was recorded only in 8.0% and 7.3%, respectively. Pollen extracts of CD and MP were used for skin tests on 63 patients at Thiruvananthapuram, a place far away from the Himalayas. However, the lowest skin positivity to BU pollen extract was at Delhi while the highest was 15.5% from Chandigarh, in the Himalayan foothills.

The average skin positivity in all patients tested at all the different centers (1+ to 3+ grade) varied from 5.8% against BU to a maximum of 23.8% against MP (Table 4). However, marked skin positivity (2+ and 3+) was highest against CD extract (7.5%), followed by 5.7% against MP and 4.7% and 4.2% against QI and BU antigens, respectively. Most of the patients showed multiple sensitizations to different tree pollen, possibly due to their cross-reacting proteins.

Nature of symptoms and seasonality of positive skin tests

Three skin test positive patients to AN, suffering from allergic rhinitis and bronchial asthma, had seasonal symptoms. Of the 11 skin test positive patients to BU extract, 7 were suffering from asthma and allergic rhinitis, and 3 had allergic rhinitis alone. As many as 5 patients showed seasonal symptoms.

Table 4 Results of skin prick tests (average) with extracts of Himalayan tree pollen in patients with respiratory allergy from four medical centers

Pollen type	Total patients tested	Skin reactivity			1+ to 3+		2+ to 3+	
		1+	2+	3+	No.	%	No.	%
<i>Alnus nitida</i>	141	10	3	0	13	9.2	3	2
<i>Betula utilis</i>	258	4	10	1	15	5.8	11	4.2
<i>Cedrus deodara</i>	531	50	37	3	90	16.9	40	7.5
<i>Mallotus philippensis</i>	425	77	21	3	101	23.8	24	5.7
<i>Quercus incana</i>	342	11	13	3	27	7.9	16	4.7

Table 5 Analysis of the binding percentage of specific IgE antibodies to Himalayan tree pollen extracts estimated by ELISA in the sera of skin test positive patients as compared to controls

Antigen used	No. of sera tested by ELISA	% Binding				Positive sera > 15%	Correlation with SPT (%)
		0-15%	15-30%	30-60%	> 60%		
<i>Alnus nitida</i>	5	0	-	5	-	5	100.0
<i>Betula utilis</i>	11	0	4	3	4	11	100.0
<i>Cedrus deodara</i>	38	9	4	11	14	29	76.3
<i>Mallotus philippensis</i>	20	10	3	2	5	10	50
<i>Quercus incana</i>	16	3	2	6	6	14	87.5

SPT = skin prick test

A total of 9 and 6 skin positive patients with CD antigen were suffering from allergic rhinitis and asthma, respectively, whereas the rest of the 25 subjects were suffering from both (Fig. 1).

The skin positivity against pollen antigens varied with age and gender of the patients tested (Fig. 2). Male patients showed a higher positivity compared to females. Similarly 21 male and 5 female subjects showed markedly positive reactions

against MP pollen extract. It was also observed that a higher number of adults showed marked skin positivity compared to children. However, the differences were not statistically significant ($p > 0.05$).

Allergen specific IgE antibodies

The allergen specific IgE antibody titers against all pollen are analyzed in Table 5. Allergen specific IgE (> 15% binding) was obtained in as

Table 6 Sensitivity and specificity of the ELISA test compared to the skin test with Himalayan tree pollen extracts

Antigens	Skin test positive		Skin test positive		Sensitivity %	Specificity %
	ET	EN	ET	EP		
<i>Alnus nitida</i>	3	0	138	13	100.0	91.4
<i>Betula utilis</i>	11	4	247	6	100.0	97.6
<i>Cedrus deodara</i>	40	9	491	150	79.2	76.6
<i>Mallotus phillipensis</i>	20	10	399	40	66.7	90.9
<i>Quercus incana</i>	16	2	325	92	88.9	77.9

ET-ELISA tested; EN-ELISA negative; EP-ELISA positive

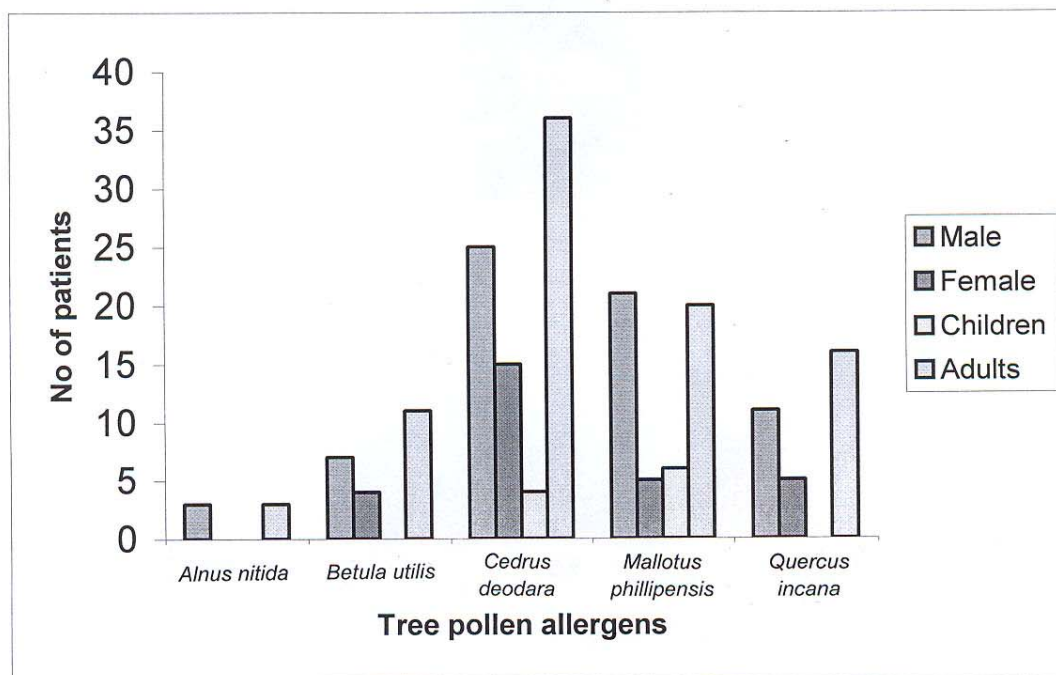


Fig. 2 Gender and age distribution of the patients showing markedly positive skin reactions against different Himalayan tree pollen extracts.

much as 100% of the cases with marked skin positivity to BU and AN pollen extracts. Sera from 7 patients recorded more than 30% binding to BU and were considered to have statistically significantly raised serum IgE ($p < 0.05$). Similarly CD specific IgE binding was observed in 11 patients with 30%-60% binding, while 14 sera out of 38 tested showed even greater than 60% binding with the CD extract. All 76.3% of the serum samples screened by ELISA showed raised allergen specific IgE ($> 15\%$ binding) to CD extract. Out of a total of 20 sera tested against MP pollen antigen, only 7 subjects had significantly raised ($> 30\%$) specific IgE. A total of 50% of the serum samples had more than 15% binding with MP antigen as compared to the controls. The presence of antibodies against QI antigen was estimated in 16 sera. Twelve of the sera showed significantly raised ($p > 0.001$) specific IgE against QI extract. Thus a correlation of 87.5% was observed between *in vivo* (skin test) and *in vitro* (ELISA) tests used for recognizing offending allergens.

Sensitivity and specificity of skin prick testing and ELISA showed that all of the skin test positive patients showed allergen specific IgE, thus a 100% sensitivity was recorded for AN and BU antigens (Table 6). A high sensitivity to CD and QI pollen was also observed (79.2% and 88.9%). However, the lowest specificity of (77.9%) was observed for QI and the highest (97.6%) for BU pollen extract.

DISCUSSION

Pollen grains constitute important components of the air causing allergies in atopic populations.^{1,3,5,9,19} Types and concentration of pollen allergens vary in different geographical regions of the world depending on the regional and local vegetation. The Indian climate supports varied and rich vegetations in different parts of the country. Therefore, the aerial pollen diversity also varies from one part of the country to another, and consequently the symptomology of the allergic patients is also diverse in different parts of the country.⁴

It has been observed that many patients residing near or far from the Himalayas with a clear history of allergy do not show positive skin reactions against a panel of routine pollen extracts. This raised the suspicion that there might be some other aller-

gens responsible for the allergic symptoms in those atopic subjects, specific to the local environment. The length of exposure to inhalant allergens affects the scope of symptoms in the local population.²⁰ Although, studies on pollen as allergens have been carried out in Delhi and other parts of the country with reference to respiratory allergy,^{15,22} information on sensitization to airborne pollen from the Himalayan region has been lacking, though a sizable population is exposed to various airborne pollen and are likely to be sensitized.

Five tree species i.e. *Alnus nitida*, *Betula utilis*, *Cedrus deodara*, *Quercus incana* and *Mallotus philipensis* were selected for clinico-immunologic evaluations, based on the reported prevalence of their pollen in the air.¹⁰⁻¹³ These taxa are important components of the native vegetation and contribute significantly to the aerial pollen flora of this region. The pollen of AN, BU, CD and QI are also reported to drift to far away places like Delhi and Kolkata, 500 km away from the Himalayas.²³⁻²⁵ Certain species of *Quercus*, *Alnus* and *Betula* are also reported to be highly allergenic in other parts of the world, particularly in Europe and the USA.^{26,27}

Sensitization patterns to these tree pollen have been assessed by skin prick test and by demonstrating reagenic IgE antibodies to the respective antigens in the sera of skin test positive patients. Skin positivity to AN was recorded in 5.9% and 14% of the patients at Chandigarh and Delhi, respectively. The higher sensitivity (marked positivity of 2+ above) observed at Delhi could be due to the more sophisticated referral hospital as compared to Chandigarh and secondly many of the patients tested at Delhi included residents from the Himalayan region with a prolonged history of allergy. AN seems less allergenic compared to *A. incana* (alder) which is prevalent in Europe.^{3,26} The higher skin reactivity at Delhi was expected compared to Kolkata, as pollen of BU were reported in aerobiological investigations from time to time in the air of Delhi.²⁵ Long distance transport of birch pollen and its allergenicity has also been reported by Wallin *et al*²⁷ from France.

CD pollen extracts showed marked skin positivity in patients from Chandigarh, followed by Kolkata and Delhi. The high skin positivity to CD at Chandigarh, which is located in the foothills of Hi-

malayas where CD forests occur naturally, could be due to the natural exposure to a higher amount of CD pollen. Although CD trees do not grow naturally in Delhi and Kolkata, their pollen have been reported to drift to these cities from the Himalayas.²⁵ However, sensitivity at Thiruvananthapuram located far south from the Himalayas could be due to cross-reactive pollen from other members of Pinaceae. Skin sensitivity among patients who are exposed to low levels of allergens or even among unexposed patients may also occur as reported by Iacovacci *et al.*²⁸ Cross reactivity among the members of the same family is reported by several studies.^{29,30} The phenomenon of cross reactivity with pollen of other members of the family Pinaceae, is suspected as trees of *Pinus* spp. are planted in parks and gardens outside their natural habitat and release pollen into the local environment.

MP pollen induced an average sensitivity in 5.2% of the atopic patients from different parts of the country, and are new potential aeroallergens. MP is a cosmopolitan genus and is part of the vegetation all over India. Skin sensitivity at Kolkata, Chandigarh and Thiruvananthapuram is explained on the basis of their plantation in different parts in the country in addition to the Himalayan region. However, MP does not form a part of Delhi's vegetation, therefore poor sensitization in this population is expected. Several members of the family Euphorbiaceae (*Mercurialis annua*, *Putranjiva roxburghii* and *Ricinus communis*) are well investigated and known for their cross reactive properties not only in India but also in Europe.^{21,31} Partial sensitization to MP in Delhi could therefore be suspected due to cross-reactivity to the pollen of *Putranjiva* and *Ricinus communis*, which are predominant allergens of Delhi.

High skin reactivity with specific IgE antibodies in patient serum against QI antigen has been observed in patients at Chandigarh compared to low sensitivity at Delhi. Mixed oak (*Quercus*) and conifers forests constitute the major portion of vegetation in the entire lower and middle Himalayas. Therefore a higher sensitization in the Chandigarh population is obvious due to the high pollen exposure. QI pollen assessed for the first time for their allergenicity in the country have been found to be important allergens of northern India. However, other species of *Quercus* (*Q. ilix* and *Q. alba*) are also reported to be allergenic and are highly cross reactive with birch

pollen in Europe.³ Sensitization to oaks is established to be high in the USA and other western countries.^{6,32,33}

An analysis of sensitization patterns in male and female patients revealed that males seem to have a higher rate of sensitization than females. A hypothesis could be that males are more exposed to the outdoor environment than females who are predominantly indoors in India. A higher positivity among males to Timothy pollen is also reported from Scandinavia.²² The preponderance of allergen sensitization in males over females agrees with a reported higher prevalence of atopy as well as allergic diseases in males than females observed in different countries. Many of the patients are reported to have symptoms during the pollen season or exaggerated symptoms in months following the pollen season. It is in conformity with these findings that the prevalence of hay fever symptoms together with positive skin tests was significantly higher in exposed vs less exposed communities.³³

An analysis of skin prick test results in two age groups, i.e. upto 30 years and those above did not show any appreciable difference in sensitization patterns. Most of the allergens showed a relatively mild influence of age on the patients as also observed by Ezeamuzie *et al.*³⁴

The high percentage of sensitivity and specificity of the skin prick test and the reactive antibodies make them a reliable tool for assessment of allergies. A correlation up to 100% between skin positivity and raised allergen specific IgE was recorded with different Himalayan pollen extracts. The search for elevated levels of specific IgE antibodies against all the pollen extracts studied confirmed the presence of reagenic antibodies in the sera of these patients to respective pollen extracts. Clinical and immunologic evidence providing a good correlation between skin prick testing and specific IgE proved these Himalayan Taxa as important allergens in the Indian population. High serum levels of specific IgE were recorded in patients with marked skin positivity and a good correlation between ELISA and skin prick test against pollen allergens was also reported by other authors.^{5,35} A relatively low correlation between skin positive patients and the presence of raised specific IgE antibodies against MP antigen, as observed by

us, was also reported by Somville *et al.*³⁶ This could be due to poor exposure to MP pollen in these patients. Allergically important protein fractions of these pollen have been analyzed by immunoblot analysis and will be published separately. A detailed investigation on cross-reactivity of tree pollens from the Himalayas with that of the plains in different parts of the country will provide better insight into the sensitization to Himalayan pollen in subjects residing in the plains.

It was concluded that sensitization to *Alnus nitida*, *Betula utilis*, *Cedrus deodara*, *Mallotus philipensis* and *Quercus incana* pollen was reported in patients residing near or far from the Himalayan region. They are considered important allergens for respiratory allergy in India. High skin reactivity to pollen extracts of *Cedrus deodara*, *Mallotus philipensis* and *Quercus incana* at Chandigarh were considered important to populations residing in the hills and foothills of the Himalayas. Similarly at Delhi *Alnus nitida*, *Betula utilis* and *Cedrus deodara* had a high frequency of skin test positivity. Sensitization against these antigens in other parts of the country is attributed to suspected cross reactivity. This study will hopefully help to improve diagnosis and therapy of patients with naso-bronchial allergy.

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