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*Short Communication*

# Specific immmunoglobulin-E responses to fractions of *Cynodon dactylon* pollen as allergen in *in-vitro* diagnostic test in Kashmir Himalaya-India

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**The study was aimed at identifying the presence of specific IgE antibody levels *in vitro* to antigen. Based on positive skin test with *Cynodon dactylon* and elevated levels of total IgE (>325 IU/ml), 104 patients were selected. Healthy, asymptomatic individuals (25) with low total IgE (<325 IU/ml) were included as controls. The mean OD values by ELISA for specific IgE were 0.67±0.21, 0.57±0.18 and 0.56±0.18 with whole pollen antigen, 46-37 and 36-32 kD fraction, respectively. The specificity and sensitivity between skin test positivity with whole pollen antigen verses fraction with mol.wt 46-37 kD was 90 and 90% and for fraction with mol.wt 36-32 kD was found to be 81.1 and 89.4%. The clusters with mol.wt 46-37 and 36-32 kD may be useful in *in-vitro* diagnostic test. Fractions within these clusters need to be identified for a higher specificity.**

**Key words:** Immmunoglobulin E, hole pollen antigen, fraction.

## NTRODUCTION

Asthma is a chronic airway disease affecting 100 million people world wide with increasing prevalence each year. In Indian population the prevalence of asthma is 10%-15%, whereas in Kashmir 20-30% are suffering from respiratory allergy symptoms in Urban areas. In semi-urban areas, 29.7% are suffering from chronic bronchitis and 18.8% from asthma. Currently, the reagents, which are used for diagnosis are crude extracts of the allergen. These reagents are non-specific in nature, lack sensitivity, specificity and cross reactivity. Specific and appropriate major allergen(s) may be useful as monospecific reagent(s) for diagnosis. Fractions with molecular weights 46-37 and 36-32 kD of *Cynodon dactylon* pollen were demonstrated to be major allergens in a study conducted at this center. The present study was aimed at estimation of specific IgE antibody levels *in vitro* to the identified antigens.

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## MATERIALS AND METHODS

### Subjects criteria

Of the patients attending the Allergy Clinic at Allergic Clinic Department of Immunology and Molecular Medicine, SKIMS, Srinagar, 104 of them who had a confirmed diagnosis of bronchial asthma were studied. The criteria used to confirm atopic bronchial asthma in these patients were clinical symptom combined with elevated total serum IgE levels and positive skin test responses. Patients below 12 years, above 60 years and pregnant women were excluded from the study. The consent of every patient was taken prior to skin testing and serum collection.

### Skin test

Pollen antigens selected based on the local aerobiological calendar were used for skin testing by intradermal injections (Cure well India Ltd). Intradermal injection of buffer saline acted as negative control

and histamine phosphatase as positive control. Immediate and late phase cutaneous responses were recorded at 20 min and 6-8 h after allergen challenge, respectively.

### Clinical studies

Based on intradermal skin test positivity of *Cynodon dactylon* pollen, 104 patients were selected for the present study. Skin test was not performed for 25 asymptomatic healthy individuals. Serum samples were collected from the patients and healthy individuals and were stored at -20°C until further use.

### Pollen extraction

The proteins were extracted from the pollens of *Cynodon dactylon* as described by Sridhara et al. (1992). Pollens collected were defatted with diethyl ether. The extract was prepared in 0.05 M ammonium bicarbonate buffer, pH 8.0 by continuous stirring for 18 h on a magnetic stirrer at 4°C. The extract was centrifuged (10,000 rpm, 4°C) and the supernatant brought to 90% saturation with ammonium sulphate. The precipitate was dissolved in distilled water and dialysed using "Spectropor" membrane (Molecular weight cut off 2,000). The dialysed extract was passed through filter membrane.

### Total IgE

The Total IgE levels were estimated by ELISA using a commercial kit (General Biologicals Corp, Taiwan). Anti-IgE coated wells were used to detect the levels of total IgE. Patient's sera 20 _l and controls were added into appropriate wells. One hundred microlitre of zero buffer was dispensed in each well and mixed thoroughly for 10 s and incubated at room temperature for 30 min. Washing was carried out 5 times with distilled water and 150 _l of anti-IgE enzyme conjugate was added and gently mixed and incubated for 30 min at room temperature, wells were washed 5 times and 100 _l of tetramethylbenzidine (TMB) solution was added and then incubated for 20 min. Reaction was stopped by adding 50 _l of stop solution to each well and read the optical density at 490 nm with a micro titer plate reader within 15 min.

### Specific IgE

ELISA was performed to measure the *Cynodon dactylon* specific IgE levels in individual patients sera with whole pollen antigen and also with its fractions by the method of Voller et al. (1980). Antigenic extracts were diluted in carbonate bicarbonate coating buffer pH 9.6 and 100 _l per well (0.1 _g/100 _l) was added. This was followed by overnight incubation at 4°C. After washing thrice with phosphate buffer saline (PBS) tween 20, the plates were incubated with 1% BSA for 2 h at 37°C. After washing thrice with PBS tween 20, the plates were incubated with 100 _l (1:100 diluted in PBS pH 7.4) individual patient's sera for 2 h at 37°C. As a control, normal healthy human serum was also tested, after washing thrice with PBS tween 20. The Anti-IgE, conjugated to horse radish peroxidase-HRP (Sigma Co), was added to each well (1:2000 diluted in PBS pH 7.4) and incubated for 2 h at 37°C followed by thrice washing with PBS tween20. 100 _l of enzyme substrate O-phenylenediamine dihydrochloride was added in 0.05 M phosphate citrate buffer containing 30% $H_2O_2$ and was added to each well. The plates were incubated for 30 min at room temperature. The colour development was stopped by adding 50 _l of 5 M $H_2SO_4$ to each well of microtiter plate. Absorbance was measured at 490 nm. Sera showing ≥3 times the O.D. compared to negative control was considered as ELISA positive.

### Sodium dodecyl sulphate polyacrylamide gel

#### *Electrophoresis (SDS-PAGE)*

SDS-PAGE was performed with the extracted proteins, using a vertical slab gel apparatus (Bio-rad, laboratories) as described by(Laemmli,1970). Electrophoresis was carried out in a discontinuous buffer (0.025 M Tris, 0.2 M glycine and 0.1% SDS) system. Antigen samples were diluted in sample buffer and boiled at 100°C for 3 min. Extract was loaded in concentration of 5 _g/well on 6%, 15% stacking gel and resolving gel. Samples were allowed to run for 5-6 h at 100 V and 25 m A. Molecular weights were estimated with the use of standard molecular weight protein markers.

### Electro elution

Portions of the gel containing the separated fractions were cut and processed for electro elution by employing the method described by (Andersen and Heron,1993). Electro elution was done using a 2 mm phosphate buffer, pH 6.8. This buffer was chosen because it provided effective elution associated with a minimal generation of heat and found non-toxic for pollen antigen. Gels were equilibrated for 40 min in equilibrium buffer after the termination of the electrophoretic run during which removal of toxic products SDS, Tris, Glycine and also swelling of the gel occurred. The electro elution of the gel was performed for 20 min, with a 40 V current. After the termination of the electro elution, the separated components were harvested. Fractions were kept frozen at -20°C until use.

### Statistical analysis

The mean, standard deviation, specificity and sensitivity were calculated for the aforementioned parameters.

## RESULTS

The specific serum IgE levels were performed by ELISA method. The mean O.D values for specific serum IgE levels in 104 patients with whole pollen antigen of *Cynodon dactylon* and its fractions with molecular weights 46 to 37 and 36 to 32 kD were observed to be 0.67±0.21, 0.57±0.18, 0.56± 0.18 and in normal's it was found to be 0.13±0.03, respectively . The specificity and sensitivity between the skin test positivity with whole pollen antigen verses fractions were performed and it was observed that for fraction with mol.wt 46-37 kD it was 90%, and 81.1 and 89.4% were found for fraction with mol.wt 36-32 kD, respectively. These aforementioned results are supported by western blot analysis, which showed a positive band at 43 kD in the allergic patients.

## DISCUSSION

IgE triggers the release of chemicals that are responsible

for inducing the symptoms of asthma (Satish Kumar et al., 1999). A genetically predisposed individual can develop an IgE immune response only after environmental exposure to the offending antigen (Thakur, 1989). Currently, diagnosis of allergic diseases is based on the typical history of allergic symptoms, clinical and serodiagnostic tests. It is the major isotype of anaphylactic antibodies, and although theoretically IgG4 can also be a reagin, its clinical importance is not significant. Thus *in vivo* and *in vitro* tests used in the diagnosis of allergic diseases are directed towards the detection of free or cell-bound IgE (Shivpuri, 1971). Immediate hypersensitivity skin test used to identify specific IgE sensitization is influenced by various factors, such as systemic and topical antihistamines, which may suppress the weal and flare reaction; other medications like corticosteroids can also interfere with the test. The test must be performed on a regular basis to maintain reliability, although, interpretation of the test results is subjective but not standardized. There may be false-positive and false-negative reactions, intermittent difficulty in performing the test and anaphylaxis is a potential risk. The most important factors to be concentrated are sensitivity and reproducibility of the test (Madhuri et al., 1992). *In vitro* tests are better and invaluable in many allergy studies. Though total IgE is the original screening test for allergy, it is not 100% specific for allergic disease and has been superseded by newer specific tests. Whereas specific IgE is not influenced by any concurrent drug treatment, it can be measured *in vitro*, the specificity of the test being as high as 90% for inhaled allergens. Therefore, by screening the allergy patients for specific allergen, IgE sensitivity may help for better diagnosis (Suman Latha et al., 2004).

In the present study, patients had high IgE antibody titers towards the fractions 46-37 and 36-32 kD of *C. dactylon*. Similar observations were reported in other pollens like *Prosopis juliflora* and *Amaranthus spinosus,* fraction with mol.wt. 20kD of *P. juliflora* pollen is allergenic among six fractions identified (Thakur, 1989). While in *A. spinosus* pollen, seven antigenic protein fractions were identified by (Singh et al., 2003) In the present study, the specificity and sensitivity of skin test positivity with whole pollen antigen verses fraction with mol.wt. 46-37 kD were observed to be 90.4 and 90% and for fraction with mol.wt. (36-32 kD) was found to be 81.1 and 89.4%, respectively. (Quiralte et al., 2007) studied the Olive allergen specific IgE responses in patients with different allergenic fractions of Olive and found that ole e 1 and ole e2 are the major allergens, which can be used for both *in vitro* and *in vivo* diagnostic purposes. On *C. dactylon,* there are no reports available.

*In vitro* testing for the diagnosis of allergy was acceptable as frontline diagnostic tools. Hence the results of this preliminary study on pollen fractions with molecular weights 46-37 and 36-32 kD distinctly demonstrates that these fractions may be used as *in vitro* diagnostic reagent preparations. However, there is a need to confirm this observation by testing patients positive to pollens other than *C. dactylon* and also to identify the specific fraction(s) within these clusters.

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