Identification of IgE- binding pollen protein from *Cannabis sativa* in pollen-hypersensitive patients from north Pakistan

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Abstract: *Cannabis sativa* (*C.sativa*) is well-known for its medicinal, industrial and recreational use. However, allergies in relation to *Cannabis sativa* (*C.sativa*) are rarely reported. *C. sativa* is one of the common weeds found in Pakistan and its pollen grains are common in spring and fall season. Although categorized as an aeroallergen, there are limited number of reports regarding allergenic potential in *C. sativa*. Therefore, the current study is aimed at exploring the IgE- binding potential among the *C.sativa* pollen in local pollen allergic patients. Initial screening of *C. sativa* sensitized individuals was carried out by dot blot from the sera of pollen allergic patients. Proteins from the pollen grains were extracted and resolved on 10% gel. Eight bands were visible on gel however only one protein fragment i.e. of 14KDa size was found to bind to IgE as analyzed through protein gel blot analysis. Strong IgE affinity of a 14 kDa protein fragment from *C. sativa* pollen extract suggests its allergenic potential. Further study is required to find the exact nature of this protein fragment.

Keywords: Allergy, allergenic proteins, *Cannabis sativa*, IgE.

INTRODUCTION

Allergic disorders are a growing threat both at global and regional levels affecting over 600 million people (Sokol *et al*, 2008; Bieber and Novak, 2009). These disorders are on a constant rise in Asian countries (Gerez *et al*, 2010). Local flora and fauna, environment and the molecular makeup seems to be important factors contributing towards allergies prevalent in a particular population (Bieber and Novak, 2009). There is an ever growing number of allergy sufferers and due to the limitations in association to the current therapeutic and diagnostic modalities there is a need for developing more effective and specified therapeutic and diagnostic modalities. The current therapies including the use of anti-histamines and corticosteroids are generally effective, however there are reports showing that the patients responded well towards the allergen specific immunotherapy (Reha and Ebru, 2007; Incorvaia *et al*, 2013). Similarly, the current traditional diagnostic tools rely on the mixture of allergenic and non-allergenic proteins which may lead to further sensitizations or in case of prior sensitization the worst case would be anaphylactic shock. Specific immunotherapy aims to target the underlying cause of the disease with long lasting effects (Ronborg *et al*, 2012). Therefore, this study aimed at identifying the IgE binding protein from the C. sativa pollen grain.

Aeroallergens are the most inevitable class of allergens. Among them pollens and house dust mites are the most common outdoor and indoor allergens in Asia (Moorcroft *et al*, 2006; Majkowska *et al*, 2007; Gerez Lee *et al*, 2010; Abbas *et al*, 2012). Local flora at a particular time of year is the main source of seasonal pollen allergy. In Pakistan, unfortunately the list of all the local allergens is far from complete and very few types of pollens have been characterized for their allergenecity. *Parthenium hysterophorus, Populus euphractica, Broussonetica papyrifera, Dalbergia sissoo, Eucalyptus globules, Prosopis juliflora and Lantanacamara* are few names of allergy causing plant species in Pakistan (Hinz *et al*, 2006). Many other allergens are yet to be identified. Our study focused on *C. sativa* (Hashish). It is a common grass weed belonging to the family Cannabaceae, locally known by many names like hemp, marijuana, bhang, ganja and hashish. It is the most abused illicit drug. Dried parts of the plant are often smoked as cigarettes. Unlike most of the developed countries it grows wild in various parts of Pakistan, especially being abundant in the north-west area of Pakistan (Bousquet *et al*, 2007; Majkowska Pelka *et al*, 2007; Abbas *et al*, 2012). It is responsible for causing allergic disorders in spring and fall season (from March to October) (Ong and Leung 2006). *C. sativa* pollens occupy a large proportion of the total pollen count in the air. It is one of the recently established common aeroallergen in Asia. However, so far there is only a hand full of reports about the allergy cases due to *C. sativa* caused by inhalation during smoking, injection or even ingestion (Stockli and Bircher 2007; De Larramendi *et al*, 2008). *C. sativa* has already been established as potential source of aeroallergy in Japanese and Spanish population (Tanaka *et al*, 1998; Gamboa *et al*, 2007). In Pakistan, the first report of *C. sativa* as a threat to atopic patients came in 2009 (Abbas *et al*, 2009). In the current study we have confirmed the allergenic potential of *C. sativa* in Pakistani population and identified the potential allergenic protein.

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Characterizing the protein fragment would be helpful for the purpose of diagnosis and for designing the therapeutic and prophylactic strategies.

MATERIALS AND METHODS

Pollen collection

Pollen grains were collected from mature anthers of *C. sativa* growing in Islamabad, from March 2010 till May 2010. Stems bearing mature anthers were separated from the plant and were allowed to dry at room temperature over clean surface. Upon drying, anthers released the yellow colored pollen grains which were collected. The identification was confirmed under light microscope (Medo Lab Inc, Sioux City, America). The procedure was adopted from study done by Rahl (Rahl, 2008).

Crude extract preparation

This was done in two steps as described by Rawat (Rawa *et al*, 2004) and Mandal (Mandal *et al*, 2008). Pollen grains were defatted in order to remove the lipids by adding diethyl ether (Reanal Finechemical Co, Budapest, Hungary) that is 3-4 times the volume of pollen grain sample. Defatted pollen was suspended in 1:10 (w/v) in phosphate buffer saline (pH 7.2) at 4^{o}C (Haier, Japan) by continuous stirring for 20 hours in magnetic stirrer (VELP Scientifica, Milano, Italy) using 1 inch bar. This sample was then centrifuged in centrifuge machine (Eppendrof, Hamburg, Germany) at 12500g at 4^{o}C for 40 minutes. The supernatant was then filtered through 0.2µm filter (Sartorius Stedium Biotech, Goettingen, Germany) and was collected in a sterile vacutainer (Axygen Scientifica, California, USA) which were then stored at -20^{o}C (Haier, Japan) for further use.

Total protein estimation

Total protein content of pollen samples was determined by Bradford Assay using Bovine Serum Albumin (BSA) (Merck KGaA, Darmstadt, Germany) as standard. The protocol was adopted from study done by Bradford (Bradford, 1976).

Characterization of crude extract

Sodium dodecyl sulphate polyaccrylamide gel SDS-PAGE (BioRad miniprotean Tertra cell, Singapor) was used to characterize the crude extract of *C. sativa* allergens. 10% gel was used in conjunction with tris glycine running buffer (Vivantis, Selangor DE, Malaysia) pH 8.3 at 120V. Afterwards the gel was stained with coomassie brilliant blue staining solution (MP Biomedical, France) followed by destaining in glacial acetic acid (Sharlau Chemie, Spain), methanol (Sigma Aldrich, Germany) and distill water solution. This procedure was adopted from Laemmli (Laemmli, 1970).

Immunoassay of crude extracts

For further proceeding, aero allergy patients visiting National Institute of Health (NIH), Chak Shehzad, Islamabad, Pakistan were randomly selected from November 2009 to February 2010 (655/12/NCVI/Visits). All patients included in the study were above 5 years of age. The consent of patient or their guardian was obtained prior to include them in study. Their detailed medical history was taken before their selection into the study. The criterion which was set for the allergic patients to be included in the study is given below:

a) Well established allergic asthma/ allergic rhinitis/ conjunctivitis/hay fever.
b) Symptoms being exaggerated upon exposure to pollen sources (pollen producing plants)
c) The patient must not have taken any prophylactic or therapeutic anti-allergic treatment particularly antihistamines at least since last 3 days before coming for skin prick test.

Whereas inclusion criteria for control group are given below:

a) The subject must be healthy with no signs, symptoms or history of any type of allergic disorder.
b) Must not be on any type of anti-allergic regimen (as there are many pathological conditions apart from allergy in which anti histamines are suggested).

Skin prick tests (SPT) against few of the common aeroallergens (Broussonetia papyrifera, Thresher, Raw Cotton, Dust Mites) prevalent in Pakistan was done at NIH. Histamine was used as positive control while normal saline was used as negative control. To prick the skin, sterile lancets were used. The result was allowed to develop for 20 minutes. Diameter of 3mm and above was considered to be positive. Patients showing positive results were selected for the study. Same procedure was done with healthy individuals. Those showing negative result were selected as control group for further proceeding.

Serum samples

Approximately 5ml blood was collected in plain vacutainers (BD Franklin Lakes Nj, USA) from both the experimental and control group, it was centrifuged at 8000rpm for 5min. The separated serum was collected in sterile vacutainers and stored at -20 for further use.

Dot blot assay

Dot blot assay was done to screen the pollen allergy patients against *C. sativa* allergy by method described by Hawkes (Hawkes, 1982). Nitrocellulose membrane (BioRad, Singapore) 1x1 was treated with 8 µl allergen extract, dried for 1 hour at room temperature and incubated for 1 hr in 3%BSA blocking solution (Merck KGaA, Darmstadt, Germany). Then treated twice with TTBS (Tween 20- Tris Buffer Saline) (Scharlu Chemie, Sentmenat, Spain), 10 min per wash and was incubated for 1 hr in the primary antibody (sera of patient and healthy individual respectively) solution. Washing step was repeated and the membrane was incubated for 30 min in the anti-human IgE peroxidase conjugate solution (Gene Tex, USA). Washing step was again repeated. The

membrane was then developed for 5min in the TMB (3,3',5,5'-Tetramethylbenzidine) substrate (Autobio diagnostics, China).

Table 1: Presence of 14kDa protein of *C. sativa* in pollen allergic patients (1s-5s) and healthy controls (1c-5c)

	Serums	14kDa protein
1	Pool 1s	+
2	Pool 2s	+
3	Pool 3s	+
4	Pool 4s	+
5	Pool 5s	+
6	Pool 1c	-
7	Pool 2c	-
8	Pool 3c	-
9	Pool 4c	-
10	Pool 5c	-

Western blotting

Based on the results of dot blot assay, patients showing positive results against the *C. sativa* crude extract were divided randomly into 5 groups. Each group comprised of fifteen patients. The serum of each group was pooled and was used as source to detect allergen specific IgE primary antibody. Similarly, individuals comprising the control group were also divided in 5 sub-groups (12 individuals per group). A serum from individuals in each sub group was pooled and was used for the identification of allergen via western blot. Protein bands on SDS-PAGE were electrophoretically transferred to the NC membrane by using semi dry technique and procedure adopted from Towbin (Towbin *et al*, 1979). After blotting for 1 hr, unbound sites on the NC membranes were blocked by incubating them with 3% BSA. TBST was used as washing agent. After washing the NC membrane strips they were incubated with pooled sera of dot blot positive patients whereas the pooled sera from healthy volunteers showing negative skin reactivity and dot blot were used as control. After washing, the strips were incubated with anti-human IgE peroxidase conjugate (1:1000). TMB (tetramethylbenzidine) solution was used as substrate for developing the results.

RESULTS

Microscopic Analysis of pollen grains

Pollen grains from *Cannabis sativa* collected from mature anthers of *C. sativa* were analysed under light microscope and their size was roughly found to be 60µm in diameter with morphology like a monocolpate pollen grain as shown in fig. 1a.

Protein quantification from pollen extract

Total protein estimation of the crude extract of pollen grain of *Cannabis sativa* pollen grains was done by using Bradford assay using Bovine serum albumin (BSA) as standard. The total protein content was found to be 98 µg per 100µl. This protein extract was found to be 9.8% of the defatted pollen (taken as starting material) which was 1mg.

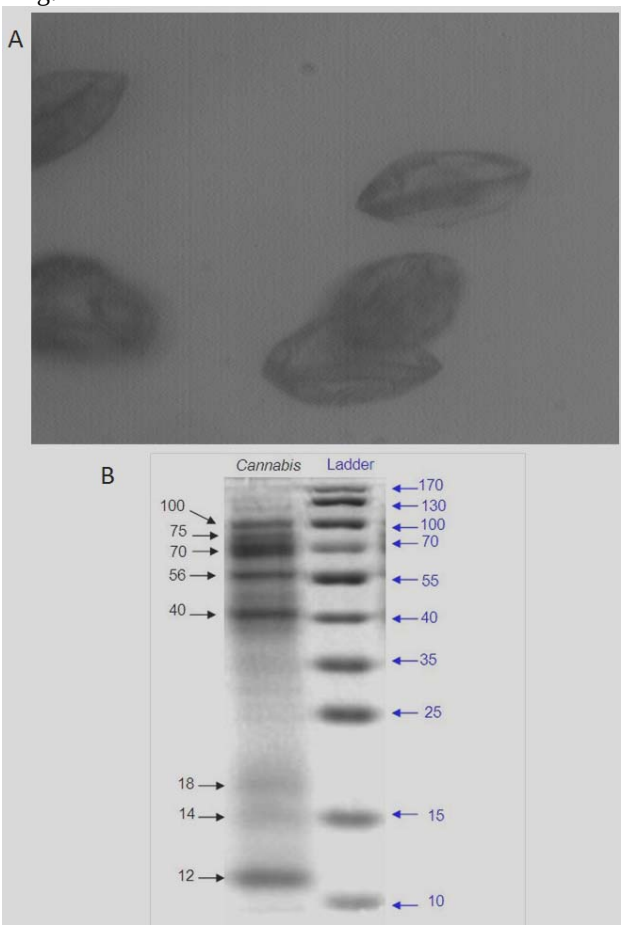

Fig. 1: Microscopic view of the Pollen grains of *Cannabis sativa* and the immunoblot showing its extracted proteins. a) Microscopic analysis of pollen grains of *Cannabis sativa* through a light microscope (100 x). The figure shows monocolpate pollen grain of about 60µm in diameter. b) 10% polyacrylamide gel showing various peptide fragments extracted from pollen grains of *Cannabis sativa*. Right lane: Pre-stained protein ladder used as reference; Left lane: Proteins extracted from *C. sativa* pollen grain resolved into 8 protein bands with size ranging from 12kDa-100 kDa.

Resolution of proteins from the crude extract on SDS PAGE

SDS PAGE of crude extract of *Cannabis sativa* revealed 8 protein bands with molecular weights ranging from 12 kDa to 100 kDa. Protein bands with molecular weight of 12 KDa and 40-100 kDa were very prominent and finely resolved with the exception of 14 kDa and 18kDa band which were comparatively diffused and were not very finely resolved (fig. 1).

Dot blot assay

About 100 patients fulfilling the selection criteria and positive skin prick test (SPT) were selected for screening against *C. sativa* by dot blot assay carried out on

nitrocellulose (NC) membrane. Male female ratio was 63:37. Control group comprised of 60 healthy individuals with male female ratio of 35:25. In the dot blot assay, the pooled sera of 75 pollen allergic patients (out of 100 selected patients) showed reactivity against *C. sativa.* All the individuals in the healthy control group showed negative results.

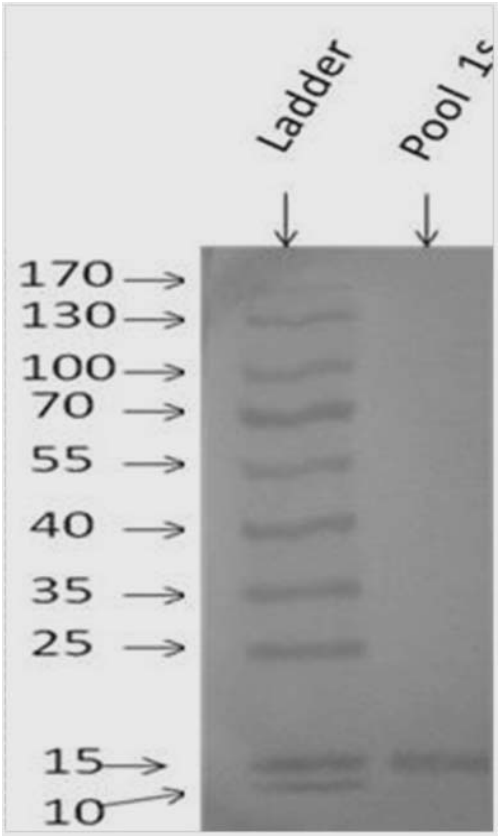

Fig. 2: Western blot showing an IgE binding protein fragment from *Cannabis sativa* crude extract. Lane 1 shows protein ladder. Pool 1s represents results of one pool of patient's sera. Anti human IgE peroxidase conjugate used as secondary antibody. 14 kDa protein identified as allergen. No band seen in control pools.

Determination of IgE-binding proteins

Western blot semi dry technique was used to identify the allergy causing protein. Total protein extracted from 1mg of defatted pollens separated in 8 fragments on SDS PAGE and were transferred to NC membrane. These strips were used as a probe to capture antigenic fragment from the pooled sera of allergic patients. Later incubation with anti-human IgE peroxidase conjugate as secondary antibody detected a 14 kDa protein band. In all the 5 pools of allergic patient's sera an IgE sensitization for 14 kDa fraction was observed. No bands were observed in the control group. This suggests that 14 kDa peptide fragment has strong binding affinity towards IgE (fig. 2 and table 1).

DISCUSSION

There is a rapid rise in cases of allergy since last decade (Abbas *et al*, 2009). In Pakistan, weather conditions support the diverse range of trees, herbs, shrubs and grasses. Especially in the spring season i.e. from March till May, there is an alarming rise in pollen concentration in air (Pawankar *et al*, 2008) posing a threat for the atopic patients.

Pollen allergy being the most inevitable form of allergies ranks second highest cause of allergy after dust mites in Pakistan (Ong and Leung, 2006). Being light weight, pollens are able to spread to a wide area causing allergies. As observed under the microscope the pollen grains of *C. sativa* is 60µm in size and are monocolpate. Generally the particles of larger size (greater than 20µm) get deposited in the nasopharynx or oropharynx causing allergic rhinitis, conjunctivitis etc while the smaller particles (e.g. 8µm or smaller) can penetrate the respiratory tract and cause allergic response in that anatomical area. Reports show that *C. sativa* is a clinically important aeroallergen which can have a causal role in the induction of allergic symptoms (Lindemayr and Jager, 1980; Stokes *et al*, 2000; Mayoral *et al*, 2008, Incorvaia and Barbara, 2013, Mari, 2001). *C. sativa* has been reported to be one of the major culprits responsible for the prevalent respiratory allergic disorders in the fall season in Pakistan (Pawankar *et al*, 2008). Identification of allergenic protein component is an important milestone for designing targeted therapies. Therefore, in view of the abundant growth and clinical significance of the wild weed i.e. *C. sativa*, the allergy causing component was identified in its pollen proteins. The pollen proteins from *C. sativa* were extracted and resolved on 10% gel showing eight protein fragments of following sizes: 12 KDa, 14 kDa, 18 KDa, 40 KDa, 56 KDa, 70 KDa, 75 KDa, 100 KDa (fig. 1). Allergic patients especially atopic patients generally exhibit sensitization against multiple allergens as reported by Mari (Mari, 2001), therefore a group of 100 individuals known to be allergic towards pollens were randomly selected, pooled and screened for IgE-sensitization against protein from *C. sativa* pollen grain. ¾ pooled sera from allergic patient's revealed sensitization against *C. sativa* pollen protein in dot blot. Further analysis for the identification of allergen in these patients was carried out through western blotting which revealed a 14 kDa fragment to be a potential allergen in local population. Various allergens have been identified and characterized from the *C. sativa* leaf, roots and flower extracts, however there are not many studies reporting the allergenecity from the pollen extracts. In the current study, we reported a 14kDa long peptide fragment from the *C. sativa* pollen protein extract as a possible causal agent for sensitization against *C. sativa* pollen (fig. 2 and table 1). The allergenic potential of 10 and 14kDa long peptide fragments from *C. sativa* has been previously

reported (Gamboa *et al*, 2007; Tanaka *et al*, 1998). Racial and ethnic differences accompanied by molecular and genetic differences may contribute towards differences in the sensitization patterns (Joseph *et al*, 2000; Gamble *et al*, 2010).

A recent study characterized the allergenic components of different parts of *C. sativa* such as leaves, buds, flower and roots. Interestingly, the peptide fragments from the enzymes involved in the primary metabolism of plants, such as ribulose-1,5- bisphosphate carboxylase/ oxygenase (RuBisCO), oxygen-evolving enhancer protein, ATP synthase, phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase, were reported as allergens (Nayak *et al*, 2013). IgE sensitization was observed with different subunits of RuBisCO enzyme and it is possible that 14kDa fragment represents one of the smaller subunits of RuBisCO. However this requires further investigation (Nayak *et al*, 2013). RuBisCO is found frequently in plants due to its essential role in the rate limiting step in photosynthesis. Its allergenic potential has been reported in various plants previously (Vrtala *et al*, 1998; Taylor, 2002; Nayak *et al*, 2013).

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CONCLUSION

This current study highlights the allergenic aspect of *C. sativa* in local pollen allergic patients. It was shown that C. s*ativa* pollen protein separates into eight different protein fragments ranging from 12 to 100kDa (12 kDa, 14 kDa, 18 kDa, 40 kDa, 56 kDa, 70 kDa, 75 kDa, 100 kDa). IgE binding with the 14 kDa protein fragment shows its allergenic potential in Pakistani population.

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