# Immunochemical comparison of the allergenic potency of spores and mycelium of *cladosporium cladosporioides* extracts by a nitrocellulose electroblotting technique

**H. Bouziane[a], J.P. Latgé[b]** and **M. Lelong[c]**

[a]Département de Biologie. Université Abdelmalek Essaadi. Faculté des Sciences. Mhannech II. Tetouan. Morocco. [b]Unité des Aspergillus. Institut Pasteur. Paris. France. [c]Service de Pédiatrie. Centre Hospitalier Docteur Schaffner. Lens. France.

## ABSTRACT

*Background:* The lack of well standardized or characterized extracts that contain the relevant allergens of the appropriate fungus is resulting in a high heterogeneity of the commercial preparation.

*Material and methods:* Immunochemical detection of the allergens composition of spore and mycelium of *C. cladosporioides* was studied by electroblotting using sera from *Cladosporium* allergic patients and 125 I- anti- human IgE. A MW range of allergens between 16 to 88 KDa was identified. The most important with a MW of 16, 20,30, 39, 43, 50, 60 and 88 KDa.

*Results:* The allergenic composition of spore and mycelium looked very similar. However, partial or total inhibition of the serum with a conidial or mycelial extract demonstrated that the total concentration of allergens (particulary 20 and 60 KDa molecules) was higher in the conidium than in the mycelium.

*Conclusions:* These results indicated that conidium and mycelium contained the same allergenic determinants but at different concentration in the two propagule. Results with 50 % inhibited sera demonstrated also that the total concentration of allergens was higher in the conidium than in the mycelium.

Correspondence:

H. Bouziane, MD
Université Abdelmalek Essaadi. Faculté des Sciences
Département de Biologie. Mhannech II
BP 2121. Tetouan. Morocco
E-mail: hasbouz@hotmail.com

***Key words:*** *Cladosporium cladosporioides*. Allergen extracts. Spore. Mycelium. Immunoblott. Cross-inhibitions.

## INTRODUCTION

Over the last years, fungal allergy has played an increasingly important role in allergy[1-3]. The lack of well standardized or characterized extracts that contain the relevant allergens of the appropriate fungus is resulting in a high heterogeneity of the commercial preparation. The allergens composition of different species of *Alternaria alternata, Aspergillus fumigatus, Curvularia lunata* and *Epicoccum nigrum*[4-7] vary according to nutritional components growth of media, temperature, incubation period and extraction method. The choice of the propagule, spore or mycelium, selected for the preparation of the best allergenic extracts is another suspected cause of the variability among commercial preparations. RAST inhibition and crossed radioimmunoelectrophoresis (CRIE) have demonstrated that allergen composition of spores and mycelia of *Alternaria, Cladosporium, Pleurotus* and *Psilocybe* differ both in quantity and quality[8-11]. Although spore may contain the most relevant allergens but in some species they are difficult to obtain. A comparison of *Alternaria alternata* spore and mycelium extract found that mycelium extract have

greater potency than that of spore preparation on the basis of skin prick test, RAST inhibition and basophile histamine release[12]. However, RAST and CRIE which rely either on the coupling of the allergens to CNBr or on intermediate rabbit antiserum do not allow a chemical identification of the allergens specific of the conidium or the mycelium. Immunoblotting is recognized as the most adapted method to identify fungal allergens[13-16]. The blotting technique provide biochemical characterization such as MW of the allergens. A nitrocellulose electroblotting technique has been used to compare and characterize the allergens of conidia and mycelia of the most allergenic species of *Cladosporium, C. cladosporioides*.

## MATERIAL AND METHODS

### Allergenic extracts

The strain LCP 404 of *Cladosporium cladosporioides* (Muséum d'histoire naturelle, Paris, France) was grown on 2 % malt agar medium in 20 cm petri dishes. After 3 to 4 weeks of growth at room temperature, The spores were harvested from the surface of dry culture media with a paint brush. The mycelium was grown for 48 h in a 2 liters Biolafitte fermenter containing 2 % glucose, 1 % peptone (Prolabo) and 0.1 % Rhodorsil 426R at 25 °C, 700 rpm, 0.5 vvm. Mycelium was recovered by filtration and washed extensively with water. Extraction of spore and mycelium allergens was performed by disruption of fungal cells suspended in 50 mM Tris pH 9.0, 1 mM EDTA and 1 % PVP as previousely reported[17]. The disrupted material was centifuged at 18000 rpm for 30 min and the supernatant was recovered and stored at –80 °C. Protein content was estimated using the Bio-Rad technique (Bio-Rad technical informations).

#### *Human sera*

Patients allergic to *Cladosporium* were selected by Dr Lelong (Service de Pédiatrie, Hopital Schaffner, Lens) on the basis of positive skin prick test. A pool of sera from patients (S, M, B, C) in the proportion 2:2:1:1 (V/V) was prepared. This pool had a RAST class 4 measured on CNBr activated cellulose paper disk coupled to *C. cladosporioides* spore extract[17].

Sera were also adsorbed with different concentrations of mycelial and conidial extracts. For absorption experiments, sera were diluted (1:1 V/V) in 10 mM phosphate buffer pH 7.2 + 0.8 % NaCl + 5 % defatted milk (Gloria) + 0.1 % Tween 20 (PBS-MT). One volume of diluted sera was added to one volume of allergenic extract and the mixture was shaken for 3h at room temperature. Two concentrations of extracts per 100 μl serum were used, respectively 100 μg and 0.25 μg of spore proteins and 100 μg and 1.25 μg of mycelium proteins. These proteins concentration gave respectively 100 % and 35 to 50 % of RAST inhibition with the pool of sera used[17]. Control sera were obtained from patients not allergic to *Cladosporium.*

### Allergen detection

#### *Electrophoresis*

The electrophoresis was carried out using a slight modification of the technique of Laemmli et al[18]. The stacking gel contained 3 % polyacrylamide in 0.125 M Tris-HCl pH 6.8, 0.1 % SDS buffer. The separation gel contained 12.5 % or 15 % polyacrylamide in 0.375 M Tris-HCl pH 8.8, 0.1 % SDS buffer. The upper and the lower reservoir buffer was 50 mM Tris, 372 mM Glycine, 2 % SDS. The sample buffer contained 625 mM Tris-HCl pH 6.8, 5 % 2-mercaptoethanol, 2.3 % SDS, 10 % Glycerol and 0.001 % Bromophenol blue as tracking dye. All samples containing 80 – 100 μg protein were heated at 100 °C for 3 min. The electrophoresis was run at 30 mA/gel (160 × 1.5 mm) for the stacking gel and at 60 mA/gel for the separation gel. Proteins standards (phosphorylase b [94 KDa], bovine serum albumine [67 KDa], ovalbumine [43 KDa], carbonicanhydrase [30 KDa], soybean trypsin inhibitor [20.1 KDa] and lactalbumine [14.4 KDa]) (Pharmacia) were used to determine the molecular weight of *Cladosporium* allergens.

#### *Electrophoretic transfer*

At the end of electrophoresis run (7 or 8 h), the proteins were transferred from the separation gel to a nitrocellulose membrane 0.45 μm (Schleicher and schuell) by electrophoretic transfer. The membrane sponge pads and filter paper(Watman n.º 3) were previously equilibrated for 5 min in the blotting buffer composed of 25 mM Tris, 192 mM glycine pH 8.3 and 20 % methanol. The nitrocellulose sheet was placed on the top of the polyacrylamide gel. They were subsequently embedded in a double layer of filter paper and sponge pad before being inserted in a trans blot cell (LKB) filled with precooled (+ 4 °C) blotting buffer. Blotting was performed at 30 V/Cm during 18 h. After transfer of the proteins, the nitrocellulose membrane was saturated for 1h at room temperature in PBS-MT.

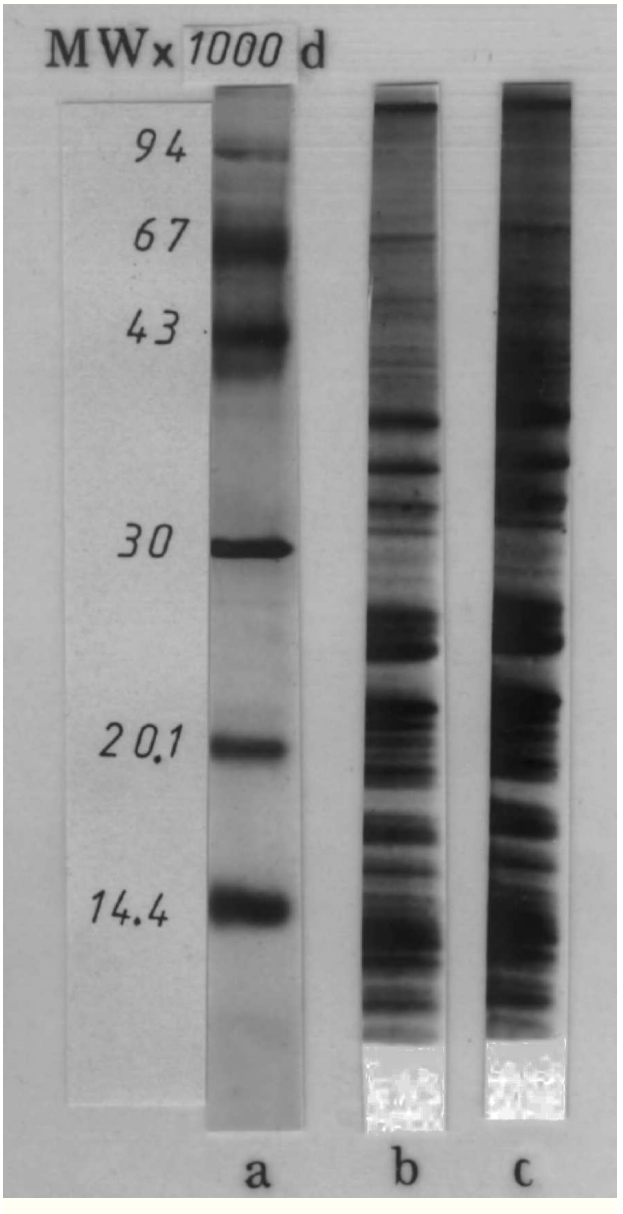


Figure 1.—Separation by SDS-PAGE of spore (b) and mycelium (c) extracts on 12.5 % polyacrylamide gel. Lane a correspond to molecular weight markers. Gel stained with CBB.

The blotted gel and a reference unblotted gel were stained with 0.25 % coomassie brillant blue (CBB) R250 in 45 % methanol and 10 % acetic acid. To estimate the efficiency of the transfer, 0.2 % Ponceau red in 3 % trichloroacetic acid was used to stain proteins after transfer to nitrocellulose[19].

*Immunoblot staining*

Nitrocellulose strips (12 × 0.5 cm) were incubated overnight on a shaker at 4 °C with the sera diluted 1:10 in PBS-MT buffer. Adsorbed and non adsorbed positive sera and control negative sera were used. The nitrocellulose strips were washed 3 times for 10 min with PBS buffer containing 0.1 % Tween 20 (PBS-T). The strips were then incubated overnight with 125 I-labelled antibodies anti-IgE (Pharmacia) in PBS-T buffer (50000 cpm/strips) at room temperature. After 6 washings for 10 min with PBS-T, the strips were dried and placed on top of an X-ray film (Kodak-pathé)and exposed in a kodak X-omatic cassette equipped with a Du-Pont cronex intensifying screen (Laborix) for detection of the 125 I-labelled proteins by autoradiography[20].

## RESULTS

When the spore and mycelium extract of *C. cladosporioides* were subjected to SDS-PAGE, over 50 bands of proteins with a MW comprised between 10 and 110 KDa were detected after CBB staining (fig. 1). No important differences were noticed in the proteic pattern of spore and mycelial extract.

Only 10 to 12 allergenic proteins were identified in the spore and mycelium preparation after transfer to nitrocellulose and incubation with the pool of positive sera and 125 I- labelled anti-IgE (figs. 2, 3). After two days of exposure in the cassette 7 allergens can be identified with a MW of 16, 20, 39, 43, 50, 60 and 88 KDa (fig. 2b, c). A prolonged exposure (up to 10 days) revealed 3 to 5 supplementary allergens with a MW of 30, 35, 41, 72 and 80 KDa (fig. 3c, d, f). Controls were negative with the exception of a slight non-specific labelling at the top of the gel (fig. 2d).

The allergenic composition of spore and mycelium extract looked similar. However, the staining intensities of several bands appear different in these two extracts and two proteins 20 and 60 KDa were detected specifically in the spore (fig. 2).

Partial inhibition of the serum with a mycelial extract (giving 50 % RAST inhibition) reinforce the staining of these 20 and 60 KDa bands (fig. 3c). However, complete inhibition of the serum with 1 mg/ml of mycelial proteins resulted in the disappearance, almost complete for the the 60 KDa band, of the spore allergens (fig. 3b). With spore extract giving 50 % RAST inhibition, the labelling of the 20 and 60 KDa bands disappaered completely (fig. 3d). Mycelial allergens transferred to nitrocellulose were also inhibited completely or partialy with spore extracts giving respectively 100 % or 50 % RAST inhibition (fig. 3e, f).

These results indicated that conidium and mycelium contained the same allergenic determinants but at different concentration in the two propagule. Re-

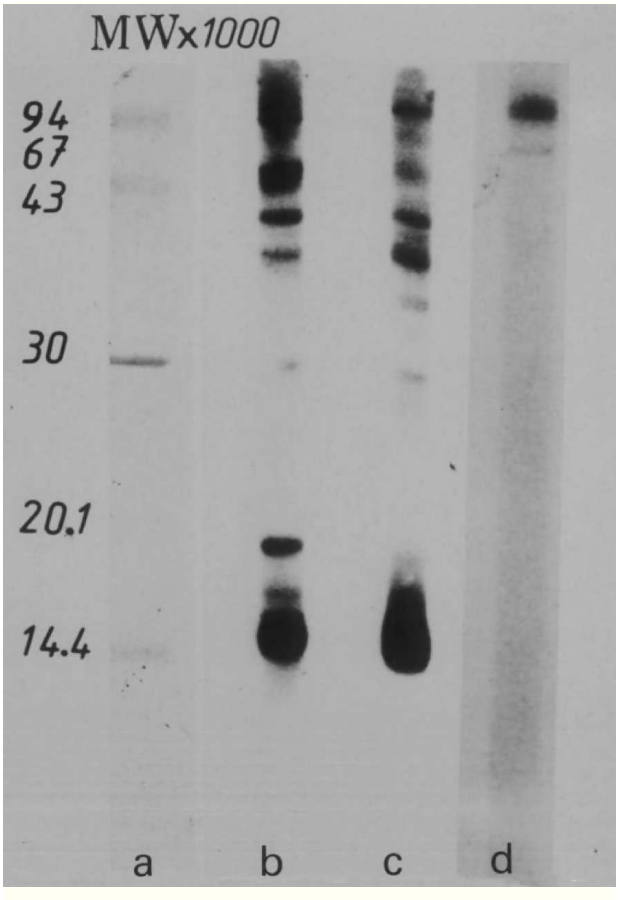


Figure 2.—Identification of IgE-binding proteins by autoradiography after 2 days exposure following SDS-PAGE on 15 % polyacrylamide gel and electroblotting. Strips a shows Ponceau red staining of MW markers. Strips b-c show respectively the transferred spore and mycelium extracts incubated with human sera from *Cladosporium* allergic patients. Strip d was incubated with the sera of non-allergic patients.

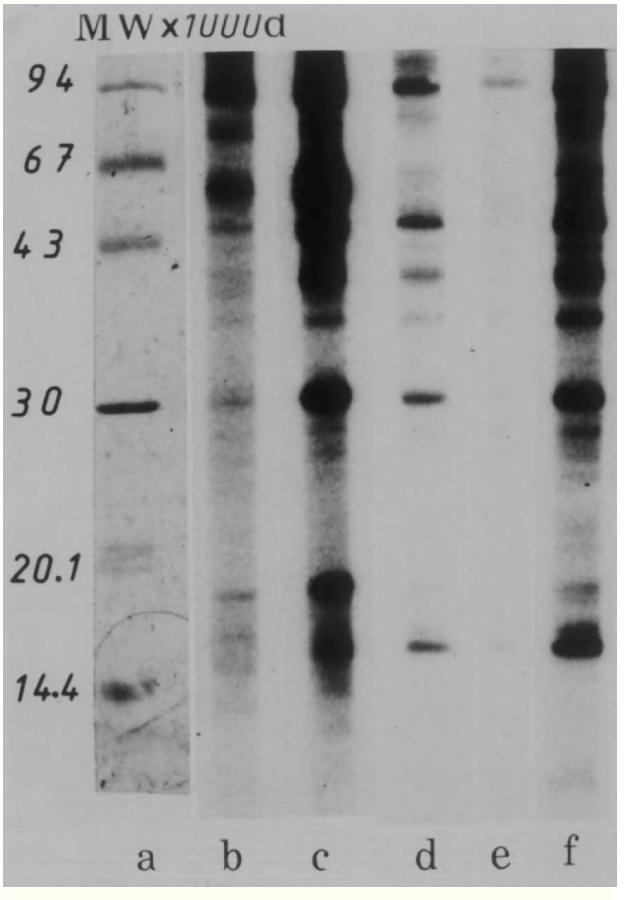


Figure 3.—Immunoblotting experiments using sera adsorbed with spore and mycelium extracts after SDS-PAGE on 12.5 % polyacrylamide gel. Spores strips (b-d) were incubated with sera adsorbed with mycelium and spore extract at a concentration giving 35 to 50 % (c) and 100 % (b) RAST inhibition for mycelium and 50 % RAST inhibition for spore (d). Mycelium strips (e-f) were incubated with sera adsorbed with spore extracts at concentrations giving similar 50 (f) and 100 % (e) RAST inhibitions values.

sults with 50 % inhibited sera demonstrated also that the total concentration of allergens was higher in the conidium than in the mycelium.

## DISCUSSION

This study and previous reports on immunotransfer methods had demonstrated the usefulness of using such techniques to identify fungal allergens[13-16,21,22]. Such method can be efficiently used to control the presence of all the major allergens in a commercial preparation[23]. In *C. cladosporioides* extracts, the same allergens were always detected among various batches of spore production. This result indicate that the extraction procedure and the buffer used are very well adapted to the recovery of *Cladosporium* allergens.

The molecular weight of fungal allergens identified in *Alternaria, Aspergillus*, *Cladosporium, Curvularia, Drechslera, Epicoccum, Fusarium, Ganoderma, Penicillium* and *Psilocybe* was generally comprised between 10 and 90 KDa[7,13,14,24-30]. A considerable allergenic relationship was found between *Cladosporium herbarum, C. cladosporioides* and *Alternaria alternata* by the immunoblot technique[31,32]. A cross-reactivity between *A. flavus* and *P. citrinum* was documented by Yu et al[33]. This evidence of the presence of shared allergenic epitopes in these fungi is different to the sensitivity of the subjects to several fungal extracts. It express similarities of different species with respect to sensitization and IgE human response. Spores and mycelium of *C. cladosporioides* have the same qualitative allergenic composition which differ only quantitatively. Previous PCA experiments in mice and guinea pigs have demonstrated an important immunologic identity among spore extracts of

*C. cladosporioides* and *C. herbarum* whereas no cross-reactivity was observed between spore and mycelium extracts of *C. cladosporioides*[17]. This result suggest that the method of immunization can be important and may lead to the recognition of different allergenic determinants by human or animal IgE. Observations of this nature emphasize the necessity when one is using an animal antiserum for the identification and standardization of allergens to assess that the animal antiserum is recognizing the same allergenic determinants as human IgE antibodies.

The allergens of *C. cladosporioides* have a MW range between 16 and 88 Kda. This is similar to others allergens detected in fungi which have the same range of MW[6,7,13,14,24-30]. Most of these allergens have a glycoproteic nature, the β-Galactofuranoside glycoconjugate part; found on conidia and conidiophore of *Aspergillus niger*[34]; being essential to confere the allergenic property to the molecule whereas the allergenic activity of the Cla h 2 (Ag 54) allergen of *C. herbarum* was not sensitive to deglycosylation[35]. The 16 KDa and the 20 KDa allergens detected in *C. cladosporioides* could probably correspond to Cla h 1 and Cla h 2 the major allergens of *C. herbarum*[24]. Cross-inhibition transfers should be performed to confirm the identity of the two allergens of *C. cladosporioides* and the Cla h 1 and the Cla h 2 of *C. herbarum*.

## REFERENCES

1. Al Doory Y, Airborne F. In: Al Doory Y, Domson JF, editors. Mould allergy. Philadelphia: Lea and Febiger; 1984. p. 27-40.
2. Latgé JP, Paris S. The fungal spore: Reservoir of allergens. In: Cole GT, Hoch HC, editors. The fungal spore and disease initiation in plants and animals. New York: Plenum Press; 1991. p. 379-401.
3. Horner WE, Helbling A, Salvaggio JE, Lehrer SB. Fungal allergens. Clin Microbiol Rev. 1995;8:161-79.
4. Curran IHA, Burton M, Muradia Zhang L, Vijay HM. Influence of culture conditions on extraction time period of *Alternaria alternata* allergens (Abstract). J Allergy Clin Immunol. 1993;91: 273.
5. Little SA, Longbottom JL, Warner JO. Optimized preparations of *Aspergillus fumigatus* extracts for allergy diagnosis. Clin Exp Allergy. 1993;23:835-42.
6. Gupta R, Singh BP, Sridhara S, Gaur SN, Chaudhary VK, Arora N. Allergens of *Curvularia lunata* during cultivation in different media. J Allergy Clin Immunol. 1999;104:8547-862.
7. Bisht V, Singh BP, Gaur SN, Arora N, Sridhara S. Allergens of *Epicoccum nigrum* grown in different media for quality source material. Allergy. 2000;55:274-80.
8. Aukrust L, Borsch SM, Einarsson R. Mold spores and mycelium as allergen sources. Allergy. 1985;40:43-8.
9. Hoffman DR. Mould allergens. In: Al Doory Y, Domson JF, editors. Mould allergy. Philadelphia: Lea and Febiger; 1984. p. 104-16.
10. Weissman DN, Halmepuro L, Salvaggio JE, Lehrer SB. Atigenic/allergenic analysis of basidiomycetes cap, mycelia and spore extracts. Int Arch Appl Immunol. 1987;84:56-61.
11. Helbling A, Horner WE, Lehrer SB. Comparison of *Psilocybe cubensis* spore and mycelium allergens. J Allergy Clin Immunol. 1993;91:1059-66.
12. Fadel R, David B, Paris S, Guesdon JL. *Alternaria* spore and mycelium sensitivity in allergenic patients: in vivo and in vitro studies. Ann Allergy. 1992;69:329-35.
13. Verma J, Sridhara S, Rai D, Gangal SV. Isolation and characterization of a major allergen (65 Kda) from *Fusarium equiseti*. Allergy. 1998;53(3):311-5.
14. Menezes EA, Gambale W, Macedo MS, Castro F, Paula CR, Croce J. characterization of allergenic fractions from *Drechslera monoceras*. J Investig Allergol Immunol. 1998;8(4): 214-8.
15. Gupta Sk, Pereira BM, Singh AB. *Ganoderma lucidum*. Partial characterization of spore and whole body antigenic extracts. J investig Allergol Clin. 2000;10:83-9.
16. Shen HD; Lin WL, Tam ME, Chou H, Wang CW, Tsai JJ, et al. Identification of vacuolar serine proteinase as a major allergen of *Aspergillus fumigatus* by immunoblotting and N-terminal amino-acide sequence analysis. Clin Exp Allergy. 2001;31: 295-302.
17. Bouziane H, Latgé JP, Mecheri S, Fitting C, Lelong M, David B. Comparison of the allergenic potency of spores and mycelium of *Cladosporium*. Allergol Immunopath. 2005;33(3):125-30.
18. Laemmli OK. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature. 1970;277:680-5.
19. Towbin H, Stachelin T, Gordon J. Electrophoretic transfer of protein from polyacry-lamide gels to nitrocellulose sheets; procedures and some application. Proc Nat Acad Sci USA. 1979;76:4350-4.
20. Tovey ER, Baldo BA. Standardization of allergens. Qualitative definitions of house dust mite extracts following electroblotting and detection of components with antibody and lectin probes. Int Arch Allergy Appl Immunol. 1984;75:322-9.
21. Cruz A, Saenz de Santamaria M, Martinez J, Martinez A, Guisantes J. Fungal allergens from important fungi imperfecti. Allergol Immunopath. 1997;25(3):153-8.
22. Shen HD, Wang CW, Chou H, Lin WL, Tarn MF, Huang MH, et al. complementary DNA cloning and immunologic characterization of a new *Penicillium citrinum* allergen (Pen c 3). J Allergy Clin Immunol. 2000;105:827-33.
23. Bisht V, Kukreja N, Singh BP, Arura N, Sridhara S. Current status of fungal allergens. Indian J Allergy Asthma Immmunol. 2003;17(1):9-19.
24. Aukrust L, Borsch SM. Partial purification and characterization of two *Cladosporium herbarum* allergens. Int Arch Allergy Appl Immunol. 1979;60:68-79.
25. Paris S, Debeaupuis JP, Prévost MC, Casotto M, Latgé JP. The 31 Kd major allergen, Alt a l 1563 of *Alternaria alternata*. J Allergy clin Immunol. 1991;88:902-8.
26. Latgé JP, Moutawakil M, Debeapuis JP, Bouchara JP, Haynes K, Prévost MC. The 18 Kilodalton antigen secreted by *Aspergillus fumigatus*. Infect Immun. 1991;59:2586-94.
27. Dixit AB, Lewis WH, Wedner HJ. The allergens of *Epicoccum nigrum* Link I. Identification of the allergens by immunoblotting. J Allergy Clin Immunol. 1992;90:11-20.
28. Horner WE, Levetin E, Lehrer SB. Basidiospore allergen release: elution from intact spores. J Allergy Clin Immunol. 1993;92:306-12.
29. Lai HY, Tam MT, Tang RB, Chou H, Chang Cy, Tsai JJ et al. c DNA cloning and immunological characterizartion of a newly identified enolase allergen from *Penicillium citrinum* and *Aspergillus fumigatus*. Int Arch Allergy Appl Immunol. 2002;127: 181-90.
30. Reese G, Horner We,Lehrer SB. Basidiomycetes allergens; characterization of epitopes with monoclonal antibodies

raised against *Psilocybe cubensis* spore extract. In: Kraft D, Sehon A, editors. Molecular biology and immunology of allergens. Boca Raton, Fla; 1993. p. 275-7.
31. Vijay HM, Burton M, Young NM. Cross-reactivity of extracts of *Cladosporium* species and *Alternaria alternata*. J Allergy Clin Immunol. 1991a;87:180.
32. Vijay HM, Burton M, Young NM, Copeland DF, Corlette M. Allergenic components of isolates of *Cladosporium herbarum*. Grana. 1991b;30:161-5.
33. Yu CJ, Chiou SH, Lai WY, Chiang BL, Chow LP. Characterization of a novel allergen, a major IgE-binding protein from *Aspergillus flavus*, as an alkaline serine protease. Biochem Biophys Res Commun. 1999;261(3):669-75.
34. Wallis GLF, Hemming FW, Peberdy JF. β-Galactofuranoside glycoconjugates on conidia and conidiophore of *Aspergillus niger*. FEMS Microbiology Letters; 2001;201:21-7.
35. Sward-Nordmo M, Paulsen BS, Wold JK. Immunological studies of the glycoprotein Ag-54 from *Cladosporium herbarum*. Int Arch Allergy Appl Immunol. 1989;90:155-61.