

Identification of Cross-Reactive Proteins amongst Different *Curvularia* Species

Ratna Gupta^{a,c} Bhanu P. Singh^a Susheela Sridhara^a Shailendra N. Gaur^b
Raj Kumar^b Vijay K. Chaudhary^c Naveen Arora^a

^aCentre for Biochemical Technology, ^bVallabhbhai Patel Chest Institute, and ^cUniversity of Delhi South Campus, Delhi, India

Key Words

Allergens · Cross-reactivity · *Curvularia* · Fungi · IgE binding · IgG binding

Abstract

Background: *Curvularia lunata* is an important inhalant allergen. The present study was undertaken to investigate the shared IgG- and IgE-binding components among seven *Curvularia* species prevalent in the aerospora. **Methods:** Seven different *Curvularia* species were grown in a semisynthetic medium for 13 days. The extracts were analyzed by SDS-PAGE, immunoblot and ELISA/immunoblot inhibition using sera from *C. lunata*-positive patients and anti-*C. lunata* rabbit serum. **Results:** Different *Curvularia* species showed 11–19 protein bands on SDS-PAGE. Proteins of 12, 20, 31, 45, 53, 78 and 97 kD were present in all the species. Eight out of 98 nasobronchial patients exhibited positive skin tests to *C. lunata* and to at least five *Curvularia* species. ELISA using these sera showed IgE binding with *Curvularia* species. Immunoblot using pooled anti-*C. lunata* sera from patients showed 5–12 allergenic proteins. Proteins of 12, 31, 45, 53 and 78 kD showed IgE binding in *Curvularia* species. Antibodies against *C. lunata* detected 6–14 antigenic proteins on immunoblot. Proteins of 31, 45 and

53 kD showed IgG binding in all the species. Proteins of 31 and 53 kD showed complete IgE/IgG binding inhibition. IgE/IgG ELISA inhibition showed dose-dependent inhibition in *Curvularia* species. *C. lunata* extract required 0.17 and 0.11 µg of protein for 50% IgE and IgG inhibition, respectively. *C. clavata* and *C. pallescens* required 10 times more protein to exhibit the same inhibition and other species required similar protein levels as those required by *C. lunata*. **Conclusions:** A high degree of cross-reactivity was observed between *C. lunata* and the six other *Curvularia* species tested. *C. lunata* and *C. senegalensis* shared maximum allergenic and antigenic components.

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Introduction

Several fungi are important allergenically in Asian and Western countries [1–5]. The allergens (proteins) derived from mycelia/spores are causative agents of type I hypersensitivity in predisposed individuals [6–8]. Various studies have shown the presence of common and unique allergens in different fungal genera and species [9–12]. The presence of cross-reactive proteins in a genus results in sensitivity to more than one fungal extract. Consequently,

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Correspondence to: Dr. Naveen Arora
Room 509, Allergy and Immunology Section
Centre for Biochemical Technology, Delhi University Campus
Mall Road (near Jubilee Hall), Delhi 110007 (India)
Tel. +91 11 7666156/7666157, Fax +91 11 7667471, E-Mail Naveen@cbt.res.in

the detailed study of IgE-binding and IgG-binding protein components within a genera would be helpful in understanding the allergenic/antigenic relationships among different species. Furthermore, the knowledge of cross-reactivity can be explored to reduce the number of extracts used for diagnosis, without compromising on the diagnostic potential.

Earlier studies with *Aspergillus* species showed significant antigenic relationships among them [12, 13]. *A. flavus* was antigenically more closely related to *A. fumigatus* than *A. niger*. However, all *Aspergillus* species induce allergic disorders. *Cladophialophora ajellio* antigens have been shown to be identical to *Cladosporium carrionii* [14]. Extensive studies on cross-reactivity amongst various species of *Fusarium* and with other fungi have been carried out [15, 16]. Proteins of 14, 19, 35, 38, 45, 50 and 70 kD were common in *F. equiseti*, *F. moniliforme* and *F. solani*. A 14 kD major allergen showed dose-dependent inhibition by the three *Fusarium* species, confirming the shared allergenicity among them [16]. These findings suggest the possibility of using extract of a single fungal species in clinical practice.

The genus *Curvularia* is an important aeroallergen in nasobronchial allergy [1, 17–19]. It is represented by about 20 species, and of these, *Curvularia lunata* is the most common. RAST inhibition showed cross-reactivity of *C. lunata* with *Alternaria*, *Helminthosporium*, *Spondylocladium* and *Stemphylium* [20]. In this study, we have characterized seven *Curvularia* species by immunobiochemical techniques. Immunoblots using sera of *C. lunata*-sensitive patients and *C. lunata*-immunized rabbits were performed to identify IgE- and IgG-binding proteins, respectively. Immunoblot and ELISA inhibition were performed to evaluate cross-reactivity between different *Curvularia* species. The study should aid in identifying the common allergenic component(s) in *Curvularia* species.

Materials and Methods

Strains and Cultures

C. lunata (strain No. 4624), *Curvularia andropogonis* (strain No. 347), *Curvularia clavata* (strain No. 1182), *C. lunata* var. *lun.* (strain No. 283), *Curvularia pallescens* (strain No. 403), *Curvularia geniculata* (strain No. 3513) and *Curvularia senegalensis* (strain No. 4740) were obtained from the Institute of Microbial Technology, Chandigarh, and the Indian Agricultural Research Institute, Delhi, India. They were grown at 28 °C in Sabouraud's broth under stationary conditions and harvested as described previously [19].

Preparation of Fungal Extracts

Spore-mycelial mass (surface growth) from *Curvularia* species was harvested on day 13. Antigen extraction was carried out at 1/50 (w/v) in 50 mM NH₄HCO₃ containing 1 mM phenyl methyl sulfonyl fluoride and 2 mM ethylenediamine tetra acetic acid [19]. The extracted proteins were precipitated to 90% ammonium sulfate and centrifuged, and pellets were dissolved in water and dialyzed against water (Spectrapore® membrane, molecular weight cutoff 3,500). Protein content was measured by Lowry's method [21] after phosphotungstic acid precipitation [22].

Skin Tests and Collection of Patients' Serum

Intradermal (ID) tests were carried out with *Curvularia* extracts on 98 patients with respiratory allergy at the V.P. Chest Institute, Delhi, India. Extracts from various *Curvularia* species were prepared in phosphate-buffered saline (PBS). The lyophilized extracts were reconstituted 1/500 (w/v) in PBS and filtered through 0.22-µm filters (Millipore, Bedford, Mass., USA). About 0.02 ml of each extract was injected intradermally into the forearm of the patient. PBS was injected as negative control and histamine diphosphate (100 µg/ml; Sigma) as positive control. The reactions were graded after 20 min by comparison to the negative control [19]. Serum from patients showing positive ID tests to *C. lunata* extract was collected with their consent. Serum from 5 nonallergic individuals (negative ID test to *C. lunata*) was also collected to be used as negative control (normal human serum (NHS)). Approval to perform skin tests was obtained from the ethical body of the institute.

Rabbit Anti-*C. lunata* Serum

Antiserum to *C. lunata* was raised by immunizing New Zealand White rabbits as previously described [19].

SDS-PAGE

An amount of 150 µg of protein of each *Curvularia* extract was resolved on 10% SDS-PAGE [23]. The separated proteins were stained with 0.1% Coomassie brilliant blue and destained.

IgG/IgE Immunoblot

SDS-PAGE-resolved proteins of *Curvularia* species were transferred onto a nitrocellulose membrane in Tris glycine buffer (25 mmol/l Tris and 192 mmol/l glycine, pH 8.0) containing 25% methanol [24]. Free sites were blocked with 3% BSA and the nitrocellulose membrane was incubated overnight with 10 ml of patient serum (1/10 v/v) or rabbit anti-*C. lunata* serum (1/1,000 v/v) at 4 °C. The membrane was washed 3 times with PBS-Tween 20 and incubated with anti-human IgE peroxidase (1/1,000 v/v) or anti-rabbit IgG peroxidase (1/1,000 v/v; Sigma) for 3 h at room temperature. The color was developed with diaminobenzidine and H₂O₂ in sodium acetate buffer (pH 5).

IgG/IgE Immunoblot Inhibition

C. lunata protein binding in patient and rabbit serum was inhibited by *Curvularia* species [25, 26]. SDS-PAGE-resolved *C. lunata* proteins were transferred to a nitrocellulose membrane. After blocking the free sites, the blotted strips were kept overnight at 4 °C with a preincubated mix of 500 µl of patient sera (1/5 v/v) or anti-*C. lunata* rabbit antibodies (1/500 v/v) and 500 µl of a protein extract of other *Curvularia* species (50 µg). For positive control, *C. lunata* blotted strips were incubated with 1 ml of patient serum (1/10 v/v) and anti-*C. lunata* rabbit serum (1/1,000 v/v). *C. lunata* blotted strips probed

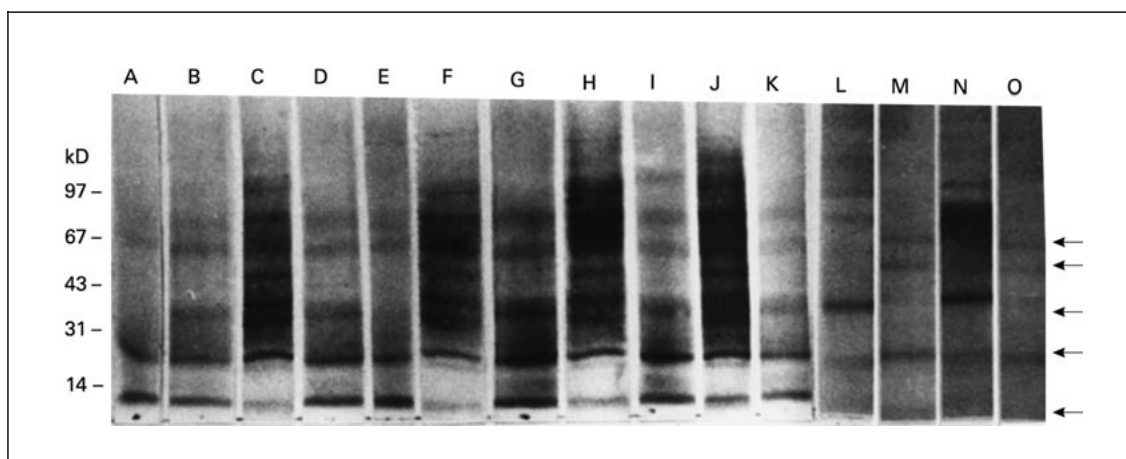


Fig. 1. Identification of IgE-binding components of *C. lunata*. *C. lunata* proteins were transferred onto the nitrocellulose membrane. After blocking the free sites, the strips were incubated with sera of 15 *C. lunata*-sensitive patients (A–O; 1/10 v/v).

with NHS and preimmune rabbit serum served as negative control for IgE/IgG immunoblot inhibition. The strips were washed 3 times with PBS-Tween 20, incubated with anti-human IgE peroxidase (1/1,000 v/v) or anti-rabbit IgG peroxidase (1/1,000 v/v; Sigma) for 3 h at room temperature and developed.

IgE ELISA

To evaluate the IgE binding activity of *C. lunata*-sensitive patient serum, (skin test positive to *C. lunata*), ELISA was performed according to the method of Towbin et al. [24]. Briefly, each well of a microtiter plate (Nunc-Immuno™ MaxiSorp™ Surface) was coated with 1 µg of *C. lunata* protein in 100 µl of carbonate buffer (pH 9.6) and incubated overnight at 4°C. After washing with PBS, the free sites were blocked with 3% BSA for 1 h at 37°C. This was followed by overnight incubation with 100 µl of individual patient's serum (1/10 v/v) at 4°C. The plate was washed and incubated for 4 h at 37°C with anti-human IgE peroxidase (1/1,000 v/v; Sigma). The color was developed with *o*-phenylenediamine. The reaction was stopped after 40 min and read at 490 nm in an ELISA reader (Dynatech).

IgG/IgE ELISA Inhibition

C. lunata extract (1 µg of protein/100 µl/well) was coated on the microtiter plate. The free sites were blocked and a preincubated mix of 50 µl of anti-*C. lunata* rabbit antibodies (1/500 v/v) or pooled anti-*C. lunata* patient sera (1/5 v/v) and 50 µl of *Curvularia* species protein ranging from 0.125 to 16 µg was added. *C. lunata*-coated wells probed with rabbit anti-*C. lunata* antibodies (1/1,000 v/v) or pooled anti-*C. lunata* sera (1/10 v/v) were the positive control (no inhibition). For the negative control, *C. lunata*-coated wells were probed with preimmune rabbit serum (1/1,000 v/v) or NHS (1/10 v/v). The plate was washed with PBS-Tween 20 and incubated at room temperature for 3 h with 100 µl of anti-rabbit IgG peroxidase (1/1,000 v/v) or anti-human IgE peroxidase (1/1,000 v/v; Sigma). The color was developed after 40 min at room temperature with *o*-phenylenediamine and H₂O₂. The reaction was stopped with 50 µl of 5 N H₂SO₄ and the optical density was measured at 490 nm.

Results

Major IgE-Binding Proteins of *C. lunata*

Immunoblot of *C. lunata* with 15 individual patient sera recognized 12- and 26-kD proteins as major IgE-binding proteins (100% of sera), whereas a 31 kD band was detected by 12 sera (80%). The proteins of 45 and 53 kD were detected by 53.3 and 40% of sera, respectively (fig. 1).

Protein Content and SDS-PAGE of *Curvularia* Species

The protein content in extracts of *Curvularia* species ranged from 25 to 45 mg/g of the spore-mycelial mass. Extracts of *Curvularia* species showed 11–19 Coomassie brilliant blue bands in the range of 14–100 kD. A prominent band at 45 kD and 12, 20, 31, 53, 78 and 97 kD proteins were visualized in all seven extracts (fig. 2).

IgE- and IgG-Binding Proteins of *Curvularia* Species

Immunoblotting with pooled patient sera showed 5–12 allergenic proteins in *Curvularia* species (fig. 3a). IgE binding was observed to 12, 31, 45, 53 and 78 kD proteins in all *Curvularia* species. A 26 kD protein exhibited strong IgE-binding activity only in *C. lunata* (table 1).

IgG immunoblot with anti-*C. lunata* rabbit serum exhibited 6–14 IgG-binding proteins in *Curvularia* species (fig. 3b). Proteins of 31, 45 and 53 kD were present in all the species, while 41 and 65 kD proteins shared by six species (table 1).

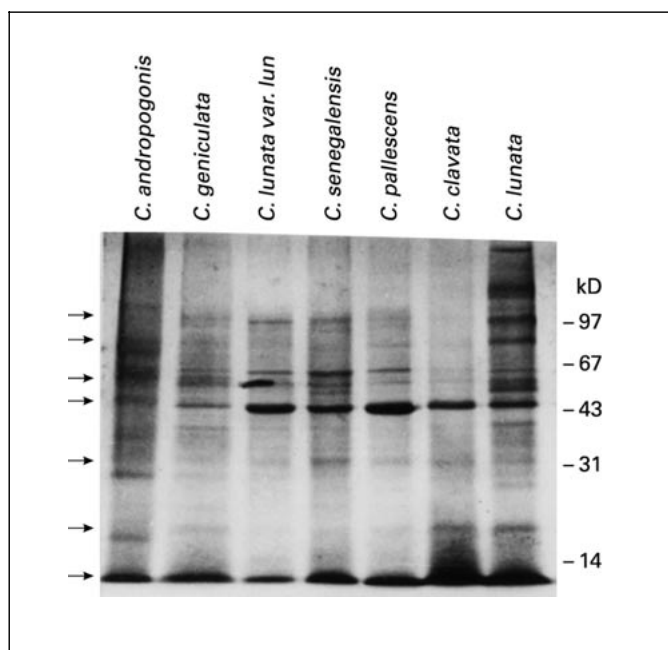


Fig. 2. *Curvularia* species on 10% SDS-PAGE in denaturing conditions.

Sensitivity to *Curvularia* Species

Extracts of *Curvularia* species were tested intradermally on nasobronchial allergy patients. *C. lunata* showed positive skin reactivity in 8.2% of the patients, followed by *C. senegalensis* and *C. pallescens* (5.1%) and other species (3.1%). *C. lunata*-sensitive patients showed marked positive ID reactivity to more than one species of *Curvularia* (table 2). ELISA with 8 *C. lunata*-sensitive patients (ID test 2+ or more) exhibited IgE binding to *Curvularia* species (fig. 4).

Allergenic Cross-Reactivity by Immunoblot Inhibition Using Patient Serum

C. lunata extract showed varied inhibition with homologous and heterologous species (fig. 5a). Proteins of 31 and 53 kD showed inhibition in all seven species exhibiting shared IgE epitopes. *C. lunata*, *C. lunata* var. *lun* and *C. clavata* showed complete inhibition of the 45 kD protein, and other species (*C. senegalensis*, *C. pallescens*, *C. andropogonis* and *C. geniculata*) could induce partial inhibition (fig. 5a). A 12 kD allergen of *C. lunata* was partially inhibited at a concentration of 50 µg with four species (*C. lunata*, *C. clavata*, *C. senegalensis* and *C. pallescens*). The *C. lunata* 26 kD protein showed complete inhibition with homologous protein and no inhibition with heterologous species.

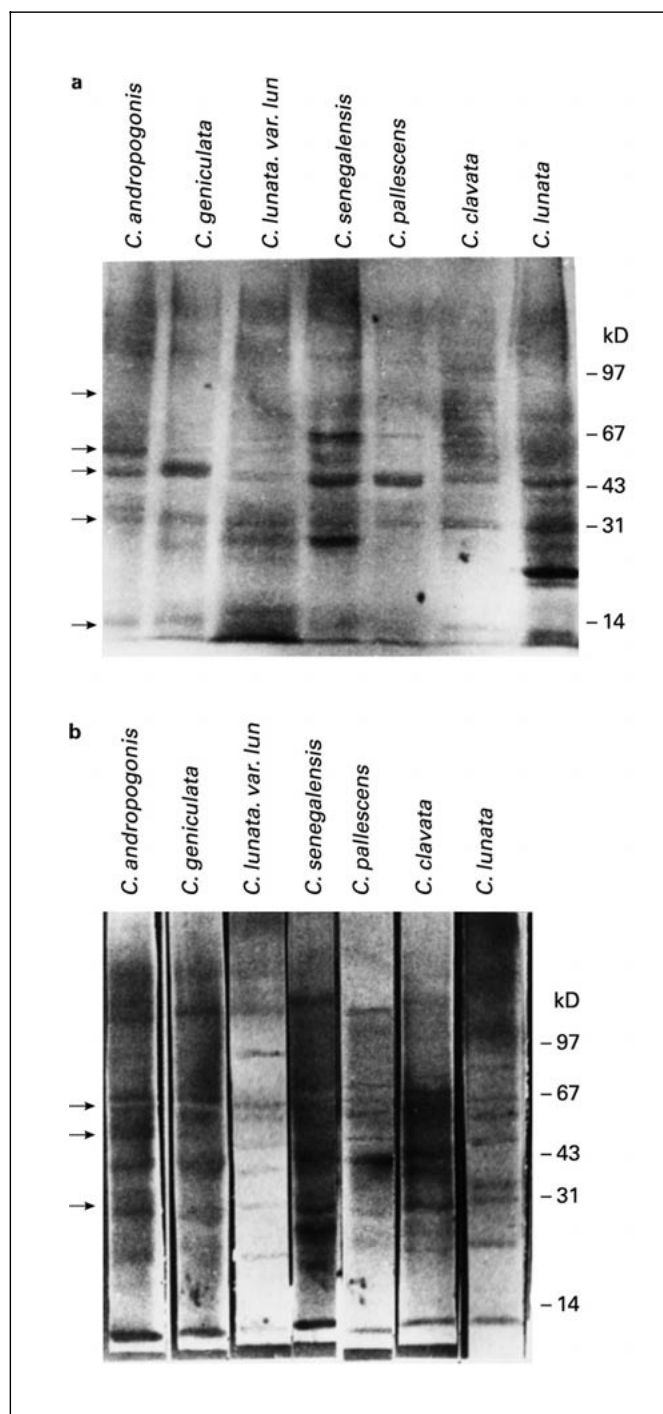


Fig. 3. a IgE-binding components of *Curvularia* species. *Curvularia* species were transferred to the nitrocellulose membrane, free sites were blocked and the membrane was incubated overnight with pooled patient sera. The membrane was washed and incubated with anti-human IgE peroxidase. **b** IgG-binding components of *Curvularia* species. *Curvularia* species were transferred to the nitrocellulose membrane, blocked and incubated with anti-*C. lunata* rabbit serum. The membrane was washed and incubated with anti-rabbit IgG peroxidase.

Table 1. Unique and shared IgE-binding and IgG-binding proteins of *Curvularia* species

<i>Curvularia</i> species	SDS-PAGE bands	IgE-binding proteins		IgG-binding proteins	
		n	MW, kD	n	MW, kD
<i>C. andropogonis</i>	17	11	12 , 30, 31 , 35, 38, 41, 45 , 48, 53 , 78 , 85	13	12, 23, 30, 31 , 35, 37, <u>41</u> , 45 , 48, 53 , 55, <u>65</u> , 77
<i>C. geniculata</i>	11	6	12 , 30, 31 , 45 , 53 , 78	7	31 , 35, <u>41</u> , 45 , 53 , <u>65</u> , 70
<i>C. lunata</i> var. <i>lun</i>	15	5	12 , 31 , 45 , 53 , 78	6	31 , <u>41</u> , 45 , 53 , <u>65</u> , 70
<i>C. senegalensis</i>	16	8	12 , 31 , 45 , 48, 50, 53 , 55, 78	9	31 , 38, <u>41</u> , 45 , 53 , 55, <u>65</u> , 70, 77
<i>C. pallescens</i>	11	5	12 , 31 , 45 , 53 , 78	6	31 , 45 , 53 , <u>65</u> , 67, 70
<i>C. clavata</i>	17	12	12 , 31 , 38, 45 , 48, 50, 53 , 55, 65, 67, 75, 78	7	30, 31 , 38, <u>41</u> , 45 , 53 , <u>65</u>
<i>C. lunata</i>	19	12	12 , 23, 26, 30, 31 , 38, 41, 45 , 48, 53 , 65, 78	14	12, 26, 27, 31 , 38, 43, 45 , 50, 53 , 55, 75, 78, 80, 88

Proteins in bold are common to all species. Underlined proteins are common to at least six *Curvularia* species. A 26 kD IgE-binding and IgG-binding protein is unique to *C. lunata*. MW = Molecular weight.

ELISA inhibition with pooled patient sera showed dose-dependent inhibition with self and heterologous *Curvularia* extracts (fig. 5b). *C. lunata*, *C. senegalensis*, *C. lunata* var. *lun*, *C. geniculata* and *C. andropogonis* required 0.16–0.18 µg of protein for 50% inhibition, indicating a close relationship between these species. *C. pallescens* and *C. clavata* required 1.1 and 1.5 µg of protein, respectively, for 50% inhibition, indicating allergenic cross-reactivity between them (fig. 5b).

Antigenic Cross-Reactivity with Rabbit Serum

IgG immunoblot inhibition with different *Curvularia* species showed complete inhibition of 31 and 53 kD proteins by all seven species (fig. 6a). *C. andropogonis*, *C. senegalensis*, *C. pallescens* and *C. clavata* showed complete inhibition of the 78 kD protein with 5 µg of protein (data not shown), whereas *C. geniculata*, *C. lunata* var. *lun* and *C. lunata* showed complete inhibition with 50 µg of protein (fig. 6a). A protein of 45 kD was partially inhibited in five species, whereas *C. lunata* var. *lun* and *C. lunata* showed complete inhibition (fig. 6a). A 26 kD *C. lunata* protein showed complete inhibition with homologous protein and no inhibition with heterologous protein.

ELISA inhibition with anti-*C. lunata* rabbit serum revealed inhibition with both homologous and heterologous *Curvularia* extracts (fig. 6b). *C. lunata* required 0.11 µg

Table 2. ID tests with extracts of *Curvularia* species on nasobronchial allergy patients

<i>Curvularia</i> species	ID reactivity of patients							
	A	B	C	D	E	F	G	H
<i>C. lunata</i>	2+	2+	2+	3+	2+	3+	2+	3+
<i>C. lunata</i> var. <i>lun</i>	2+	1+	–	1+	2+	1+	1+	2+
<i>C. andropogonis</i>	2+	1+	1+	2+	1+	2+	1+	1+
<i>C. pallescens</i>	–	1+	–	2+	2+	2+	2+	2+
<i>C. clavata</i>	3+	1+	–	2+	2+	1+	1+	1+
<i>C. geniculata</i>	2+	–	–	1+	2+	1+	2+	1+
<i>C. senegalensis</i>	1+	2+	2+	2+	3+	2+	1+	1+

A total of 98 patients were tested with seven *Curvularia* species. Out of these, 8 patients (A–H) showed marked positive ID reactivity (2+ or more). All the patients positive to *C. lunata* showed reactivity with one or more *Curvularia* species.

of self-protein, while *C. lunata* var. *lun*, *C. senegalensis*, *C. geniculata* and *C. andropogonis* required 0.12–0.18 µg of protein for 50% inhibition. *C. pallescens* and *C. clavata* required 1.5 µg of protein for 50% inhibition, indicating that they are antigenically more distinct from *C. lunata* as compared to other species.

Fig. 4. Scatter plot of ELISA values of patients sensitive to *Curvularia* species. Each *Curvularia* species was coated onto the wells of a microtiter plate and probed with 8 patients' sera. The optical density (OD) values obtained with NHS (0.18) were subtracted from the samples.

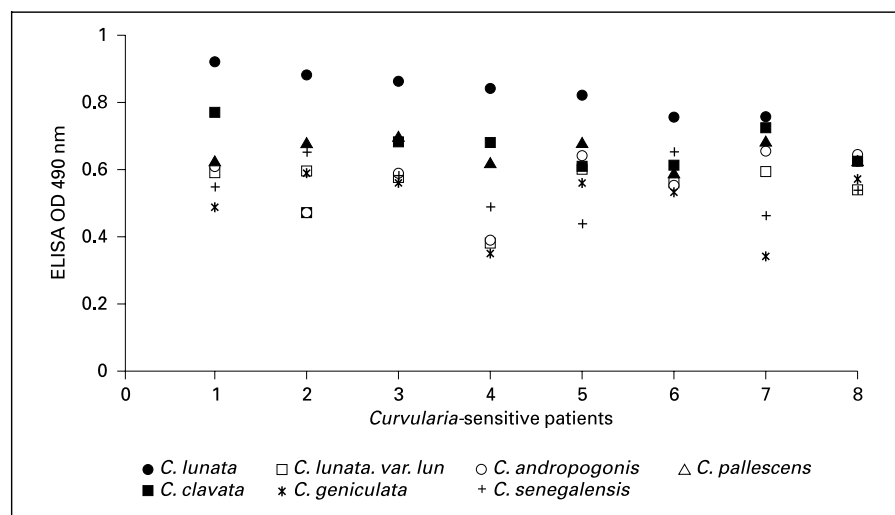


Fig. 5. a IgE immunoblot inhibition by homologous and heterologous *Curvularia* species. *C. lunata* protein strips were incubated with a mix containing 500 μ l of pooled sera of *C. lunata*-sensitive patients (1/5 v/v) and 50 μ g of *Curvularia* species. *C. lunata* proteins probed with the serum pool were used as positive control. **b** IgE ELISA inhibition with *Curvularia* species. Each well was coated with 1 μ g/100 μ l *C. lunata* antigen. After blocking, it was incubated with a mix containing 50 μ l of patient serum with *Curvularia* species ranging from 0.125 to 16 μ g/100 μ l and further incubated with anti-human IgE peroxidase. Wells probed with patient serum were used as positive control and those probed with NHS as negative control.

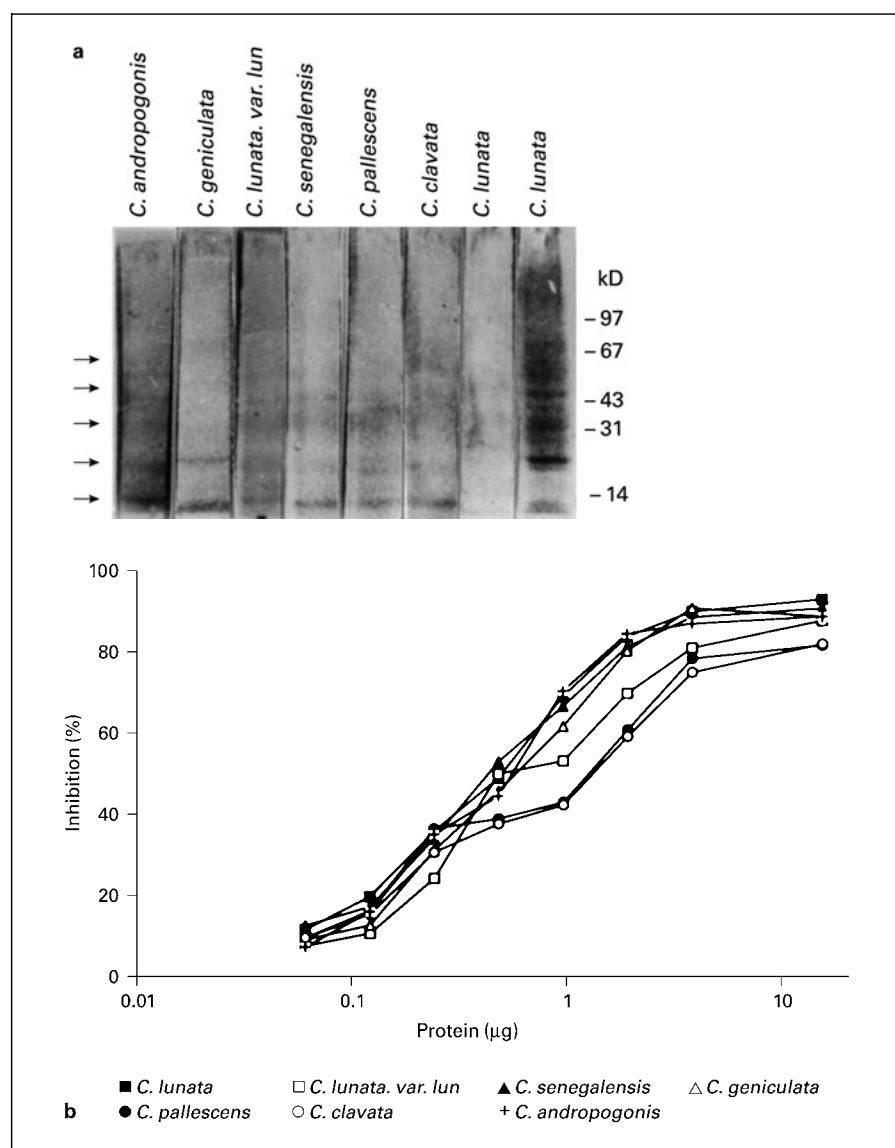
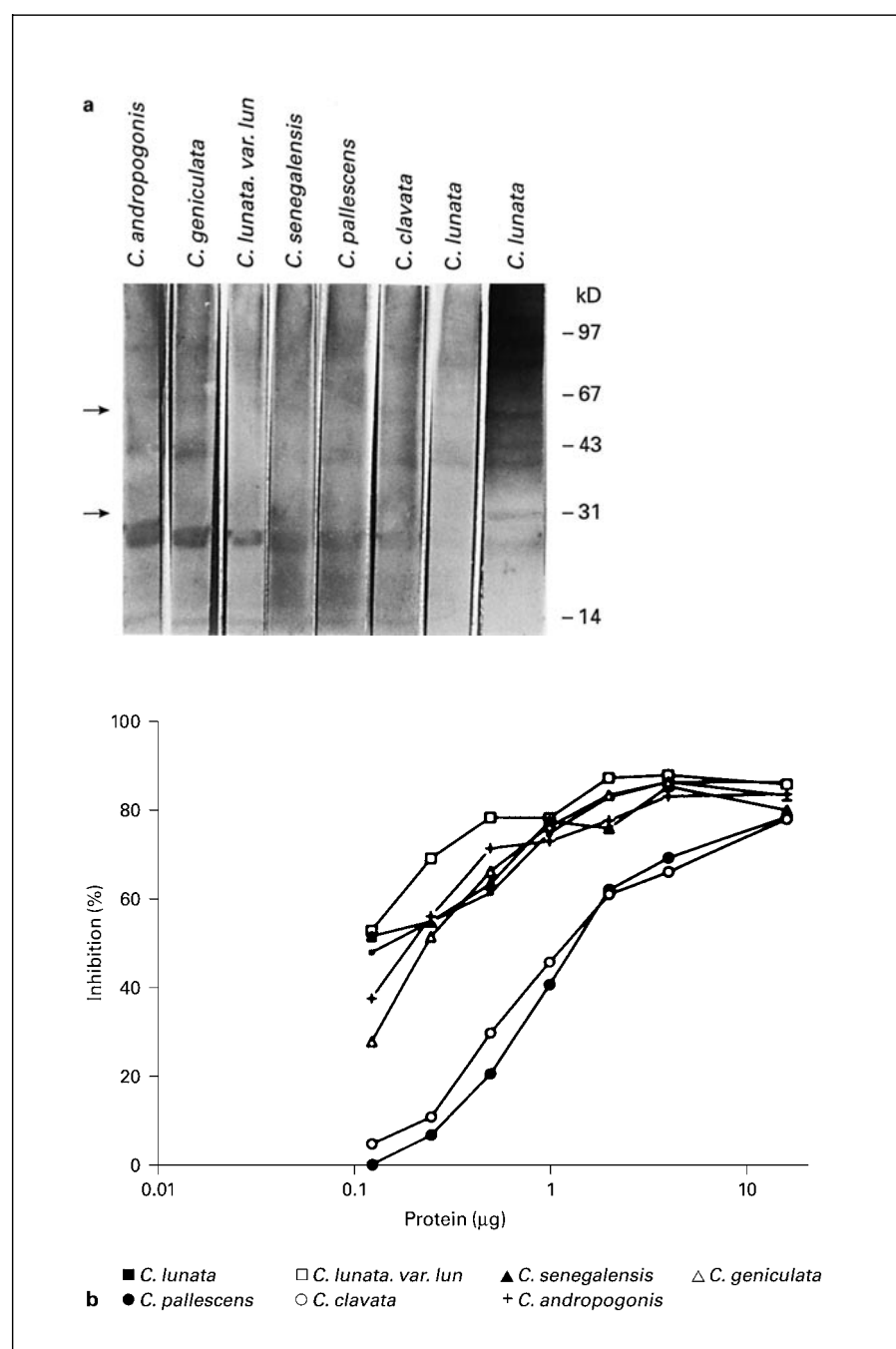


Fig. 6. a IgG immunoblot inhibition with homologous and heterologous *Curvularia* species. *C. lunata* protein strips were incubated with a mix containing 500 µl of rabbit serum (1/500 v/v) and *Curvularia* species (50 µg/500 µl). *C. lunata* proteins were probed with anti-*C. lunata* rabbit serum for positive control. **b** IgG ELISA inhibition with *Curvularia* species. Each well was coated with 1 µg/100 µl *C. lunata* antigen, blocked and incubated overnight with a preincubated mix containing 50 µl of anti-*C. lunata* rabbit serum (1/500 v/v) and 50 µl of *Curvularia* species ranging from 0.125 to 16 µg/100 µl. *C. lunata* probed with rabbit antibodies was used as positive control and with preimmune serum as negative control.



Discussion

In the class deuteromycetes, *Curvularia* is an important fungus causing respiratory allergic diseases [1–5, 17, 18]. To obtain potent allergenic material, we standardized the source material of *C. lunata* for the preparation of allergenic extracts [19]. The present study was undertaken

to identify the IgE- and IgG-binding proteins in seven common *Curvularia* species and to study the allergenic and antigenic relationships among these species.

Immunoblotting revealed different IgE binding profiles in *Curvularia* species, but proteins of 12, 31, 45, 53 and 78 kD were present in all of them and may be cross-reactive allergens (table 1). Previous studies with *Fusa-*

rium species showed proteins of 14, 19, 35, 38, 45, 50 and 70 kD as common IgE-binding proteins in *F. solani*, *F. equiseti* and *F. moniliforme* [16].

ID tests on nasobronchial allergy patients showed different sensitivity to *Curvularia* species. *C. lunata*-sensitive patients elicited marked positive ID reactivity to more than one *Curvularia* species, indicating that some proteins in *Curvularia* species are cross-reactive.

Shared IgG binding among *Curvularia* species was analyzed by immunoblot and immunoblot inhibition using anti-*C. lunata* rabbit antibodies. *C. lunata* is the commonest species; hence, antibodies were raised in rabbits against its allergens. Immunoblot inhibition with *C. lunata* antibodies showed the 31 and 53 kD proteins to be common to all the species, while few other protein components were shared by at least five *Curvularia* species (fig. 3b, 6a). This suggests the existence of similar IgG-binding proteins in different species. Shared IgG-binding determinants between *Cladosporium bantianum* and *C. carrionii* have also been shown by gel diffusion and cross-immoelectrophoresis (CIE) [14].

ELISA inhibition was performed with *Curvularia* species using anti-*C. lunata* rabbit serum and pooled patient sera to quantitate the IgG/IgE binding cross-reactivity. *C. lunata*, *C. lunata* var. *lun*, *C. senegalensis*, *C. clavata* and *C. andropogonis* required a similar amount of protein for 50% inhibition, indicating close antigenic/allergenic relationships among these species, whereas *C. pallescens* and *C. clavata* showed a distinct relationship with each other (fig. 5b, 6b). IgE binding cross-reactivity among *Aspergillus* species has also been demonstrated using a paired cross radiosorbent inhibition test. It was shown that *A. fumigatus* inhibits *A. glaucus* by 63% and *A. flavus* by 62% at 10 mg/ml concentration [12].

IgE binding inhibition of *C. lunata* proteins with other species showed that 12, 31, 45 and 53 kD allergens cross-reacted with all the species tested (fig. 5a). Many fungi contain a 45 kD protein which is a cross-reactive allergen [15]. Characterization of the 31, 45 and 53 kD proteins both at the protein and molecular level would be beneficial in studying cross-reactivity with other fungi. Studies with *Penicillium* species have shown cross-reactivity with a 33 kD protein, a major IgE-binding protein, in all three species tested [27].

These findings suggest that IgE-binding components of *C. lunata* were shared by other species. *C. senegalensis* and *C. pallescens* exhibited significant skin reactivity in *C. lunata*-sensitive patients. Also, the major allergens of *C. lunata* showed cross-reactivity with *C. senegalensis* proteins. Thus, *C. senegalensis* is antigenically and allergenically closely related to *C. lunata*. The detailed characterization of its allergens would contribute to an understanding of their role in *Curvularia* allergy.

In conclusion, significant IgE and IgG binding cross-reactivity exists among *Curvularia* species. *C. lunata* and *C. senegalensis* showed close antigenic and allergenic relationships. A 26 kD protein was unique to *C. lunata*. Proteins of 31 and 53 kD were shared allergenically and antigenically by all seven *Curvularia* species tested.

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