

Sensitization to *Ganoderma lucidum* in patients with respiratory allergy in India

A. B. SINGH, S. K. GUPTA, B. M. J. PEREIRA* and D. PRAKASH†

Centre for Biochemical Technology, University Campus, *Department of Biosciences and Biotechnology, University of Roorkee and †V.P. Chest Institute, Delhi University, Delhi, India

Summary

Although human sensitization to basidiomycete *Ganoderma* has been reported in New Zealand, North America and Europe, hypersensitivity due to this fungi is not known in India, in spite of its prevalence in the atmosphere. We have studied the atmospheric concentration of *Ganoderma* in different localities within Delhi and the sensitization level to this fungi amongst the Indian population. Aerobiological sampling, using a Burkard personal slide sampler, was carried out in Delhi for 2 consecutive years (October 1989–September 1991). The sampler was operated at 10 day regular intervals for 10 min to trap the spores. The peak season for *Ganoderma* is recorded from July to September with highest average monthly catch of 336 spores/m³ in September 1991 from south Delhi. Antigenic extracts were prepared from both, the spores and whole body of *Ganoderma lucidum*. The results of intradermal skin tests conducted on 172 patients revealed that 28.48% and 17.44% of patients showed marked skin reactivity to spore and whole body extracts, respectively. A significant correlation ($r=0.963$, $P<0.01$) was found between intradermal and skin-prick tests. More than 80% of the intradermal test positive patients had significantly ($P<0.01$) elevated IgE antibodies to the fungi in question. Thus, sensitization to *Ganoderma lucidum* has been reported for the first time in the atopic population of India.

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Introduction

Morphologically, basidiomycetes are the most complex form of fungi like mushrooms, toadstools, puff balls, stink horns, bracket fungi (polypores), smuts, rusts and jelly fungi [1]. More than 16 000 species exist according to Carmichael *et al.* [2] while Horner *et al.* [1] reported 25 000 species of basidiomycetes. High atmospheric concentrations of basidiospores have been reported from different parts of the world [3–7] and the atmospheric surveys conducted in different parts of India also indicate their prevalence in the air [8,9]. The allergenic nature of basidiospores was first suspected by Gregory and Hirst more than four decades ago [10]. Salvaggio

et al. established the relationship between a high concentration of basidiospores in the air and a high rate of asthma patient admissions in hospitals in New Orleans [5].

Later, extensive work by Lehrer and others firmly established the role of basidiospores in Type I hypersensitivity disorders using *in vivo* and *in vitro* methods [11–16]. However, recent studies conducted by other workers have further elucidated basidiospores as important aeroallergen [17,18]. The lack of information on human sensitization to airborne basidiospores in India, which supports luxuriant growth of bracket fungi during the rainy season, prompted us to carry out investigations on atmospheric concentrations and the role of *Ganoderma lucidum* in the clinical and immunological sensitization of patients with respiratory allergy in this part of the world.

Correspondence: Dr A. B. Singh, Centre for Biochemical Technology, Mall Road, Delhi University Campus, Delhi 110007, India.

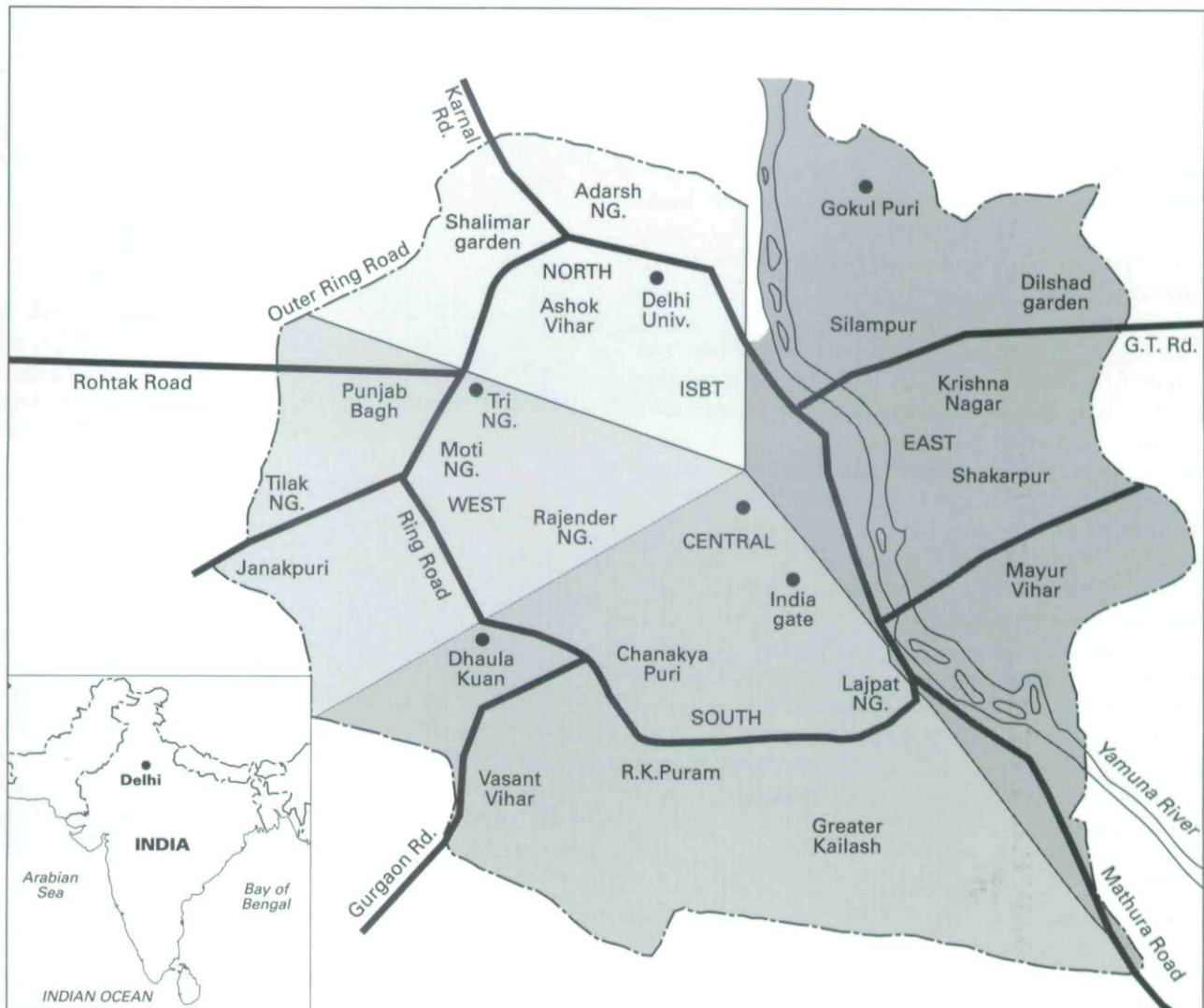


Fig. 1. Map of Delhi showing sampling zones where survey for basidiomycetes (*Ganoderma* and *Fomes*) was carried out from October 1989 to September 1991.

Materials and methods

Aerobiological survey

The concentration of *Ganoderma* basidiospores in the atmosphere of Delhi was recorded for 2 consecutive years from October 1989 to September 1991 in five different ecohabitats (Fig. 1). In brief, Yamuna Vihar in east Delhi was located in a highly populated area, Delhi university campus sites in south and north Delhi were surrounded by scrub forest and extensive vegetation, Cannaught Place in central Delhi is in the city centre surrounded by offices and shops; and west Delhi is an industrial area. The Burkard personal volumetric slide sampler was operated three times a month at each

site at a regular interval of 10 days for 10 min between 10 and 12 h of the day. Exposed slides were scanned using a Zeiss Axioscope light microscope ($10 \times 40 \times$ and occasionally $10 \times 100 \times$ oil immersion). Spore identification of *Ganoderma* and other basidiospores was based on the literature [8,19]. The spore counts are expressed as number of spores per cubic meter of air sampled (spores/m³). However, detailed methodology of sampling has been presented in our earlier publication [8].

Preparation of antigen

Collection of fruit bodies The fruit bodies of *Ganoderma lucidum*, the most commonly found species in and

around Delhi were collected from the field. The identification was confirmed based on the literature [20–22] as well as by personal communication with experts (E. Levetin, Tulsa, USA). The spores were scrapped from the upper surface of fruit bodies. Microscopic examination revealed 99% purity of the spore material used for antigen extraction. Fruit bodies with residual spores were dried at 37°C for 72 h and crushed into smaller pieces and pulverized for antigen preparation.

Extraction of antigen Pure spores and whole fruit body of *Ganoderma lucidum* were defatted before extraction. The defatted and dried materials were extracted in 1:20 (w/v) concentration in 0.05 M Ammonium Bicarbonate buffer (pH 8.1), which was supplemented with toluene and phenylmethanesulfonyl fluoride (PMSF) as antibacterial and antiprotease, respectively. The extraction was continued for 16 h at 4°C and the mixture was then centrifuged at 13 500 *g* for 20 min in cold. Supernatant was collected and dialysed (Spectraphore membrane 7000 mol. wt. cut off) extensively against distilled water at 4°C. Dialysed extract was centrifuged again and filtered through 0.45 µm millipore membrane. Clear solution was aliquoted, lyophilized and stored at –20°C. *Ganoderma* pure spore and whole fruit body extracts were designated as GSE and GWBE, respectively.

Skin tests

For skin testing, GSE and GWBE were reconstituted (1:500 w/v) in clinical grade phosphate buffer (PBS) for intradermal tests, and in 50% glycerinated PBS for skin-prick tests (1:10). The extracts for intradermal skin tests were filtered through 0.22 µm millipore membrane under aseptic conditions and were checked for sterility. Skin tests with GSE and GWBE were carried out on 172 patients with respiratory disorders. A detailed medical and personal history of these patients was taken and they were advised to stop sympathomimetic drugs 48 h before the skin tests. Intradermal (ID) skin tests were performed on each patient by subcutaneous injection of 0.01 ml of extracts on the volar surface of the arm [23]. Skin-prick tests were also performed on 48 patients (38 ID positive, 10 ID negative) to confirm and compare skin reactivity by the two methods. Fourteen asymptomatic individuals having no family history of allergy were also tested, to act as control, and to rule out any non-specific reactions. Intradermal test reactions were observed after 15 min and were graded from negative to 4+ according to the criteria of Shivpuri and Agarwal [24] and skin-prick tests as reported by Singh *et al.* [25], principally based on a weal reaction.

Serum samples

Venous blood was collected from the patients, showing either negative or 2+ and above skin reactivity. Expressed serum was separated by centrifugation at 48 *g* for 10 min before making an aliquot and storing at –20°C for further use.

ELISA

The indirect ELISA approach was followed to check the presence of allergen-specific IgE [26]. Briefly, GSE and GWBE, reconstituted in 0.1 M carbonate buffer (10 µg/100 µl) were coated onto 96 well Nunc microtitre plates. The plates were incubated overnight at 4°C. Excess antigen was washed with 0.05 M PBS (pH 7.2). Unoccupied binding sites were blocked with 3% BSA in PBS for 3 h at 37°C. After three washings with PBS (0.01% Tween 20 + 0.02% BSA), patient sera diluted (1:5 v/v) in PBS (with 0.01% BSA) were added to respective wells, except, buffer blank and control serum (pooled from 14 non-atopic controls). After overnight incubation at 4°C, washing was repeated with PBS (0.03% Tween 20 + 0.01% BSA). Second antibody, i.e. alkaline phosphatase labelled anti-human IgE (Sigma), was added (1:1000 v/v) to capture the antigen bound specific IgE. Plates were again washed with PBS (0.03% Tween 20). Antigen-serum IgE-anti IgE complex was then detected by adding pnpp (1 mg/ml in 0.1 M glycine buffer, pH 9.6 + ZnCl₂ + MgCl₂). Colour was allowed to develop and optical density (OD) was taken at 405 nm using a Nunc immunoreader.

ELISA analysis

The results of ELISA were analysed using two different approaches: (i) according to Wedner *et al.* [27] and, (ii) according to Kauffman *et al.* [28]. In brief, mean and standard deviation (SD) for OD values for buffer blank and pooled control sera was calculated which was subtracted from OD values of each sample. This adjusted value was then divided by the SD to find out the multiple of the SD. Patients having mean a OD + 2 SD, were considered positive. In the second approach, percentage binding was calculated for each sample relative to control sera and those giving a minimum of 20% binding were considered positive. These two arbitrary criteria for positivity were checked for statistical significance using the differences in OD values of controls and patients and were found significant ($P < 0.05$). The sensitivity and specificity of the assay is a function of its discrimination between signal and noise, that is, between the response at low antibody level and the

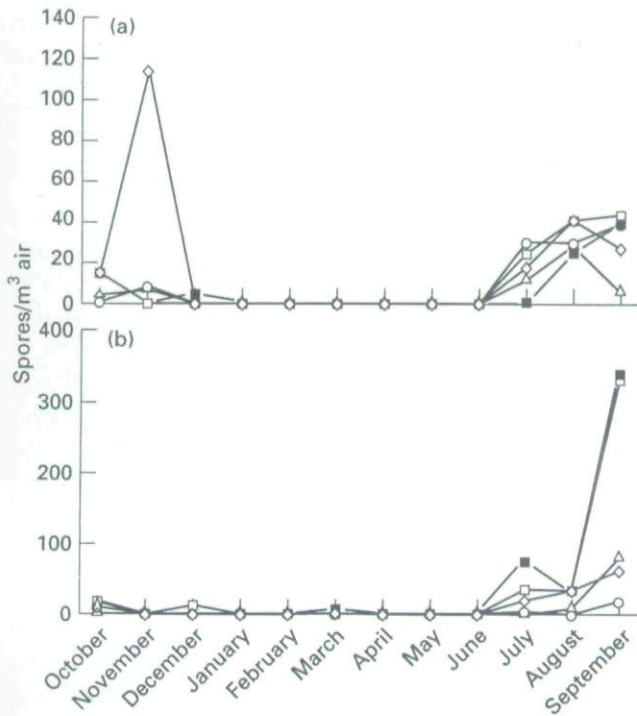


Fig. 2. Monthly concentration of *Ganoderma* basidiospores at various sites during the 2 year survey [(a) 1989–90; (b) 1990–91] at the height of 1.5 m from ground level. ■—, SD. —, CD. —x—, ND. —□—, ED. —x—, WD.

background. Therefore, sensitivity and specificity of the test were calculated as described by deBlay *et al.* [29].

Results

Atmospheric prevalence

Monthly concentration (an average of three samples) of *Ganoderma* basidiospores in different parts of Delhi during 1989–1990 and 1990–1991 are depicted in Fig. 2 (a) and (b) respectively. *Ganoderma* contributed 1% to the total mycoflora while it constituted 6% of the total

basidiospore catch in Delhi. The peak season for *Ganoderma* spores was recorded from July to September with an exceptional peak (113 spores/m³) in November 1989 from the north Dehli site. Almost similar pattern of occurrence was recorded for both the years. The highest concentration (336 spores/m³) was obtained from south Delhi in September 1991, compared with west Delhi, where the minimum concentration (17 spores/m³) was observed. The average annual concentration for different sites revealed that south and east Delhi sites had higher concentration (> 400 spores/m³), than north Dehli (133 spores/m³), central Dehli (102 spores/m³) and west Dehli (27 spores/m³).

Patient symptoms and skin reactivity

Of 172 patients (101 male, 71 female, average age 35 years) 149 had asthma, 138 rhinitis and 115 had both as categorized by clinical investigations. Only 27% reported definite positive family history, whereas 41.8% did not have any definite information. The analysis of their symptoms revealed that 21% of patients had seasonal symptoms, 17% reported perennial symptoms with irregular exacerbation, whereas 45% had perennial symptoms with seasonal exacerbations. Interestingly, a total of 19 (11%) patients reported an increase in symptoms during the rainy season and autumn, which coincided with the *Ganoderma* season in the atmosphere.

Results of intradermal skin tests with GSE and GWBE are shown in Table 1. Maximum positivity was observed with GSE in 28.48% patients, who showed marked positivity, i.e. 2+ and above, compared with only 17.44% with GWBE of the same intensity. Of 19 patients who had their symptoms during *Ganoderma* season, 47% showed marked positive reaction to GSE. None of the patients reacted with 4+ intensity to either of the extracts. The 1+ reaction was shown by 17.44% and 16.86% cases with GSE and GWBE, respectively. In all, five patients positive to GWBE were observed to have negative reaction to GSE, whereas 14 cases showing skin

Table 1. Intradermal skin-test results conducted with *Ganoderma* extracts, on 172 patients with respiratory allergy

Antigens	Total cases (n = 172)					Controls (n = 14) %
	1+	2+	3+	1+ to 3+	2+ to 3+	
GSE	30 (17.44%)	46 (26.74%)	3 (1.74%)	79 (45.93%)	49 (28.48%)	0.0
GWBE	29 (16.86%)	27 (15.69%)	3 (1.74%)	59 (34.30%)	30 (17.44%)	0.0

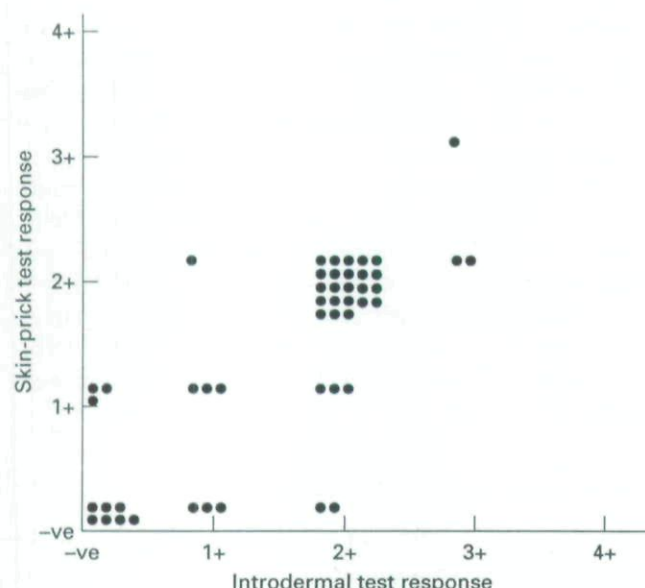


Fig. 3. Scatter diagram, showing correlation between intradermal and skin-prick test. $r = 0.96$, $P < 0.01$.

positivity to GSE were negative to GWBE. None of the control individuals reacted to either of the extract. A comparative result of 48 cases showing different grades of skin reactivity with ID and PT are shown in the scatter diagram (Fig. 3). As is evident from the figure, the higher the intensity of skin reaction the better the correlation between the two tests. A total of 82.14% of patients showing 2+ skin reaction after ID also experienced 2+ skin reactivity after PT. Of these, five patients with 2+ ID response were either 1+ (three cases) or -ve

(two cases) by PT. A statistically significant correlation ($r = 0.96$, $P < 0.01$) between the two tests has been recorded.

ELISA

Comparison of total (1+ to 3+) and marked positive (2+ to 3+) ID test reactions to GSE and GWBE among different group of patients and *in vitro* positivity (ELISA) are given in Tables 2 and 3. It was observed that almost all patients had significantly raised specific IgE to GSE in their sera. In case of GWBE, patients with allergic rhinitis and irregular perennial symptoms had a higher percentage of positivity than asthmatic patients. Nine GSE positive patients who reported their symptoms during the *Ganoderma* season also had high levels of specific IgE. When skin test negatives to GSE [17] and GWBE [27] were tested by ELISA all showed negative reactions, except two patients from each group. Both methods revealed that more than 80% patients had elevated specific IgE in their sera. Comparison of two criteria of ELISA positivity revealed a good correlation between the two methods employed. Sensitivity and specificity of our ELISA test based on results obtained by both approaches, are given in Table 4. We obtained more than 90% sensitivity and 75–85% specificity.

Discussion

Diagnosis of allergy is mostly based on identification of environmental allergen, skin tests and any *in vitro* test valid for allergen specific IgE. Sensitization to *Gandoerma*

Table 2. Results of GSE specific IgE ELISA in ID positive cases classified based on type and nature of different respiratory symptoms

Respiratory symptoms	Skin test		Sera tested	Positivity	
	1+ to 3+	2+ to 3+		ELISA* n (%)	ELISA** n (%)
Bronchial asthma	16	10	7	6 (85.7)	7 (100.0)
Allergic rhinitis	12	7	6	5 (83.3)	5 (83.3)
Asthma/Rhinitis	51	32	28	23 (82.1)	24 (85.7)
Total cases ($n = 172$)	79	49	41	34 (82.9)	36 (87.8)
Seasonal	16	14	12	10 (83.3)	12 (100.0)
Irregular	10	6	4	4 (100.0)	4 (100.0)
Perennial	43	25	23	18 (78.2)	18 (78.2)
NDH	10	4	2	2 (100.0)	2 (100.0)
Total cases ($n = 172$)	79	49	41	34 (82.9)	36 (87.8)

* According to Wedner *et al.* [27]. ** According to Kauffman *et al.* [28]. NDH. No definite history.

Table 3. Results of GWBE specific IgE ELISA in ID positive cases classified based on type and nature of different respiratory symptoms

Respiratory symptoms	Skin test		Sera tested	Positivity	
	1+ to 3+	2+ to 3+		ELISA* n (%)	ELISA** n (%)
Bronchial asthma	12	7	5	4 (80.0)	4 (80.0)
Allergic rhinitis	5	3	2	2 (100.0)	2 (100.0)
Asthma/Rhinitis	42	20	18	14 (87.5)	16 (88.8)
Total cases (n = 172)	59	30	25	20 (80.0)	22 (88.0)
Seasonal	15	12	11	7 (63.6)	9 (81.8)
Irregular	6	5	4	4 (100.0)	4 (100.0)
Perennial	30	11	9	8 (88.8)	8 (88.8)
NDH	8	2	1	1 (100.0)	1 (100.0)
Total cases (n = 172)	59	30	25	20 (80.0)	22 (88.0)

* According to Wedner *et al.* [27]. ** According to Kauffman *et al.* [28]. NDH. No definite history.

lucidum, a commonly occurring field fungi, was studied using a combination of clinical and immunological assays in nasobronchial allergy patients in India. The atmospheric survey carried out at 1.5 m from ground level revealed the prevalence of *Ganoderma* spores and the data on actual exposure risk to the population at human height. Mishra *et al.* [30] have emphasized the need for actual exposure assessment. *Ganoderma* spores occurred during late summer (rainy season) and autumn, i.e. from July to September. This pattern of occurrence is almost similar to that observed by many other workers [6,7,31,32] but the atmospheric concentration recorded in our survey is observed to be lower than reported by, e.g. Tarlo *et al.* [6] and Hasnain *et al.* [31]. This could be attributed to the urban habitat of Delhi and the time of sampling in our survey, as *Ganoderma* is known to release spores during the night or in early morning hours [6]. Our results are in best correlation with that of Levetin [32] who reports 250–1000 *Ganoderma* spores/m³ at different sites at a height of 1.5 m from the ground level. *Ganoderma* contributed 6% to the total basidio-

spore concentration recorded in this survey and constituted only 1% of total mycoflora recorded in Delhi. Hasnain *et al.* [31] also reported that urban sites will have low basidiospore counts. However, the presence of *Ganoderma* is evident from Delhi.

Quantitative variations in atmospheric concentration of *Ganoderma* within the same urban limits have been observed although not found statistically significant ($P > 0.05$). The fact that there are more parks, historical gardens and a reserve forest in south Delhi probably accounted for higher *Ganoderma* spore counts from this site.

Evidence from New Orleans [5], Ontario [6] and New Zealand [31] clearly implicate *Ganoderma* as an aero-allergen. Skin-prick test results in various reports [6,12,17] attribute 10–20% human sensitization to *Ganoderma* spores, however, during the present investigation we found a 28.48% marked intradermal positivity to GSE and 17.44% to GWBE. The difference in skin reactivity could be due to the use of ID test in our study, which is more sensitive compared with PT employed in

Table 4. Sensitivity and specificity of ELISA with respect to GSE and GWBE

Antigens	Skin test + ve		Skin-test - ve		Sensitivity	Specificity
	ET	EP	ET	EP		
GSE	41	36	17	2	89.13	89.0
GWBE	25	22	27	2	89.26	93.0

ET. ELISA tested. EP. ELISA positive.

others. We adopted the ID approach because in India skin colour is a limiting factor for skin-prick test interpretation when flare reaction is as important as the diameter of the weal. To confirm the validity of ID results we carried out PT on sufficient number of ID positive and negative patients, which correlated well with ID tests results ($P < 0.01$). Lehrer *et al.* [11] reported 11% of skin positive reactions to the spore extract of this species, whereas Butcher *et al.* [12] reported 38.1%. These differences are due to the variability in the size of the study population. Less positivity to GWBE in our study is evident as no individual can inhale the WFB and *Ganoderma* does not shed its mycelium like *Fomes* another polypore. However, skin positivity to the whole body extract suggests sensitization to fruit body regardless of mode of sensitization. Identical 17% similar skin reaction to both GSE and GWBE could be due to the presence of spore specific allergens in GWBE as whole fruit body contained residual spores in it and/or have some crossreactivity between epitopes in GSE and GWBE. Further studies on crossreactive allergens are required, nevertheless, our results suggest the role of *Ganoderma* as an aeroallergen in the Delhi atmosphere.

We performed ELISA to check the presence of *Ganoderma* allergen specific IgE in skin-test positive patients. The criteria of grading ELISA positivity is variable [33–35] and each method has its own limitations. Therefore, we have analysed our ELISA results based on mean + SD and the relative percentage binding as mentioned earlier and found almost similar results. More than 80% of skin-test positive patients were observed to have elevated levels of specific IgE in their sera against GSE and GWBE. Our results reveal that all patients, having asthma and/or rhinitis, showed good correlation between skin-test and ELISA positivity for GSE, whereas the number of such cases is low in case of GWBE. This was expected since *Ganoderma* spores, which are 5–6 µm in size are either entrapped into upper respiratory tract by nose breathing or penetrate deep into lungs by breathing through the mouth [31]. Where as mycelium alone or spores entrapped into mycelium will not be able to penetrate deeper airways because of bigger sizes. Various workers have extensively used radioallergosorbent test (RAST) to measure the basidiomycetes specific IgE in patients sera [6,11,36,37]. Although the per cent positivity in these reports is comparable with our results high sensitivity (89%) of ELISA in case of both GSE and GWBE is evident. The high specificity for GSE (89%) and GWBE (93%) suggests *Ganoderma* has less cross reactivity with other aeroallergens in India. High specificity in both cases also suggests that our extracts are potent enough in detecting individuals with cutaneous reactivity to *Ganoderma* antigens.

It is, therefore, concluded that (i) season for *Ganoderma* is July to September in India. (ii) concentration is variable in the city depending upon the amount of natural vegetation; (iii) more than 28% population is sensitized; (iv) ID tests is good for low potency antigenic extracts of basidiomycetes; (v) skin-test positive patients have elevated levels of specific IgE in their sera.

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