

Aerial fungal spectrum of Kolkata, India, along with their allergenic impact on the public health: a quantitative and qualitative evaluation

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Received: 22 December 2017 / Accepted: 4 September 2018
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Abstract Airborne fungal spores are well documented to trigger respiratory allergy. The present study provides baseline information on the quantitative and qualitative aspects of aerospores for the first time in a typical urban area of Kolkata metropolis, India. An aeromonitoring was performed by Burkard volumetric 7-day sampler for two consecutive years (August 2012–July 2014). Total concentration, seasonal variations and intradiurnal periodicities of fungal spores were estimated. Spearman's rank correlation analysis was performed using various weather parameters. The prevalence of fungal spore allergy was investigated among patients reporting to the Department of Allergy and Asthma, Mediland Diagnostics, Kolkata. A retrospective review of patients was conducted with a standard questionnaire and performing skin prick test with 20 fungal extracts. Thirty-eight fungal genera were identified with the highest spore concentration in August. Relative

humidity and rainfall were found to be positively correlated with spore concentration. A comparatively higher frequency of sufferers was observed for males and 15–35-year age group. Patients with an atopic family history and insidious onset of symptoms were prone to suffer more, and bronchial asthma was diagnosed in most. Out of 742 patients, 592 patients noticed to have positive skin reactivity toward one or more fungal extracts. *Aspergillus fumigatus* followed by *Curvularia pallescens* and *Fusarium lateritium* elicited maximum sensitization. Each of these genera also represented a considerable fraction of the total aeromycota, thereby indicating potential risk to the sensitized individuals. Further serological analysis along with the identification of fungal allergens would pave the way of immunotherapy.

Keywords Aerospore · Fungal extracts · Questionnaire · Skin prick test · Correlation · Sensitized individuals

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s10453-018-9534-6>) contains supplementary material, which is available to authorized users.

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1 Introduction

Fungal spores and mycelial cells have been known to cause adverse health effects in human (Cho et al. 2005; Simon-Nobbe et al. 2008). Compared with other allergenic sources, fungi are widespread in the environment and the exposure almost remains constant throughout the year (Crameri et al. 2014).

Atmospheric fungal spore concentration exceeds mean pollen concentration 100–1000 times (Burge 2002). Upon inhalation, airborne fungal spores or fragments of hypha may incite allergic respiratory symptoms in pre-sensitized individuals. Fungi may colonize in the human body and may damage airways by the production of toxins, proteases, enzymes (Kauffman et al. 2000) and volatile organic compounds (Dales et al. 2004). Thus, molds have a far greater impact on the patients' immune system than any other allergenic sources. Through the last four decades, a remarkable increase in various allergic diseases from 10 to 30% in India has been observed (Dey et al. 2016).

About 80 fungal genera have been stated as the mediators of airway diseases (Kurup et al. 2000). Both indoor and outdoor fungal spores trigger different respiratory troubles (Green et al. 2006). The outdoor spore concentration ranges from 230 to 10^6 spores/m³ (Knutsen et al. 2012). These also invade interiors and become major indoor allergens upon colonizing indoor substrates. Outdoor allergens have been the topic of only limited studies regarding the epidemiology of asthma (Fukutomi and Taniguchi 2015). Much remains to be studied in prevalence patterns, exposure and disease relationships, and control. The role of outdoor allergens in allergic rhinitis is noteworthy and is also rapidly increasing for causing asthma (Burge and Rogers 2000). For the proper diagnosis and treatment of the health hazards, diurnal and seasonal fluctuation pattern of airborne fungi is required (Oliveira et al. 2009). The non-viable sampling method permits the microscopic identification of fungal spores deposited on an inert surface allowing access to the total airborne fungi (Mensah-Attipoe et al. 2016). Therefore, monitoring of non-viable spores in the outdoor environment seems to be an efficient means to evaluate airborne spore load of a particular area.

The primary objective of our study was to determine both the quantitative and qualitative aspects of outdoor molds via non-viable spore sampling method. It is not always the quantity but the quality (i.e., allergenic potential) of the mold that determines the overall development of clinical allergy. That is why we have started with air monitoring for concentration analysis of airborne spores and simultaneously tested antigenic extracts of some dominant ones through SPT. The combination of spore concentration analysis

along with the determination of allergenic potential by SPT validated the platform of our study.

A 2-year-long air monitoring was conducted in Kolkata, a megacity of India, to get the quantitative features of fungal spores, and clinical investigations were performed to evaluate the qualitative aspects. Similar study has been conducted in a less urbanized zone of Kolkata (Das and Gupta-Bhattacharya 2008; Chakrabarti et al. 2012; Chakraborty et al. 2003) that differs from the present sampling site in several aspects such as population density, vegetation and pollution level. Our study is a first time effort to evaluate the aeromycoflora and their human impact in a typical urban area of Kolkata.

2 Materials and methods

2.1 Study area

Kolkata (22°33'40"N, 88°22'1"E) is one of the largest and populated (population density—24,760/Km²; ratio of population—male/female = 1000:956) cities of India. The sampling site is highly populated and congested with typical urban development. The site is surrounded by lots of buildings including housing complexes, hospitals and educational institutions, and most of the area in between is occupied by roads and streets, and slums along the streets. Like other megacities, this area is affected by high level of road traffic and gaseous and particulate emissions from motor vehicles. Vegetation in the proximity is not dense, mostly planted by man and strongly influenced by human activity. Several trees, shrubs consisting of ornamental plants and synanthropic plant communities were present near the spore trap. A variety of ruderal annuals and weeds found to grow under and between the ornamental plants.

2.2 Spore (non-viable) sampling and identification

The sampling of airborne fungal spores was carried out from August 2012 to July 2014 applying the volumetric method. A 7-day Burkard spore trap (Burkard Manufacturing Co. Ltd., Hertfordshire, UK) was installed on the top of a boundary wall of a housing complex at the height of about 2 m. Spores were collected on a special tape coated with the

mixture of vaseline and paraffin wax in toluene. The tape was replaced with a new one every week and cut into seven segments and mounted on slides with glycerin jelly. The exposed tapes were microscopically scanned by a high-resolution light microscope (Leitz, Diaplan, Germany) at 400X and counted according to the British Aerobiology Federation guidelines (1995). Several keys, atlases and pictures (Nilsson 1983; Shaheen 1989; Moubasher 1993; Samson et al. 2002) along with their appearance (color, shape) and morphology (septa) were consulted for spore identification, and identification was done up to genus levels. Spores that were difficult to be classified were grouped as 'Unknown' in the case of ascospores and basidiospores.

2.3 Study of spore concentration and spore richness

For the quantitative determination daily, monthly and annual spore concentration was estimated. The resultant slides were counted (microscopic field width 256 mm) along at 2-mm (1-h) intervals to obtain the mean hourly count, and accordingly, spore counts were calculated for daily, monthly and annual count. The spore counts were converted (conversion factor = 0.617) into spore concentration (number of spores per cubic meter of air).

To create a fungal spore calendar, individual airborne fungal genus was expressed in monthly mean concentration and presented on a particular scale, each level of which corresponded to a particular concentration of the spores.

Spore richness, i.e., number of different spores, was also counted in each month of our study years to recognize the variety of aerospores.

2.4 Study of intradiurnal periodicities

The day when the daily mean concentration of a particular spore exceeded the average mean concentration of total spore was considered as ideal to study the intradiurnal periodicity of the relevant spore type. The concentration of each hour of that particular day was divided by respective daily mean concentrations. The average data for each hour of these days were plotted to get the intradiurnal periodicity pattern of relevant taxa.

2.5 Relationship with meteorological factors

The relationships between the total as well as the predominant aerospores and climatic factors such as temperature (°C), relative humidity (%), rainfall for 24 h (mm), wind speed (km/h) and sunshine duration (h) were explored to find out any dependence. The meteorological data were obtained from Indian Meteorological Department, 4-Duel Avenue, Alipore, Kolkata-700027.

2.6 Study subjects

A cohort of 742 individuals (398 males and 344 females) was considered as study subjects. These patients were selected from the affected population visited a local allergy clinic (Department of Allergy and Asthma, Mediland Diagnostics, Kolkata) in the years 2012–2014.

Inclusion criteria were subjects with 10–65 years of age, a history of doctor-diagnosed allergic symptoms either alone or in different combination, skin prick test (SPT) grade $> +1$ using antigenic extracts of several fungal species selected on the basis of their availability and reported allergenicity. Written informed consent of each patient was signed before inclusion, and approval of the ethics committee of the clinic was obtained. Exclusion criteria were perennial or severe asthma, pregnancy or lactation, malignancy and other severe systemic diseases during skin testing. Corticosteroids and antihistamines were prohibited to avoid masking of severe symptoms.

Patients were categorized into four groups: Group A aged below 15 years (67 males and 36 females); Group B aged 15–35 years (118 males and 102 females), Group C aged 35–50 years (115 males and 95 females) and Group D aged above 50 years (98 males and 111 females).

Subjects showing negative response to all tested fungal extracts were considered as controls. Total 16 control individuals (two from each age group) were considered.

2.7 Questionnaire

The patient survey was conducted using a set of questionnaires followed by the Chittaranjan National Cancer Institute and Calcutta University, Government of West Bengal, India, following the format of Dey

et al. (2014). The questionnaire was comprised of questions on demographics (age and sex) and clinical aspects. Query on the clinical part was comprised of the description of the present symptoms, family history of atopy and nature of onset of reported symptom (sudden/insidious).

2.8 SPT

Airborne fungal spores were trapped by Andersen volumetric air sampler and impinged on a potato dextrose agar (PDA) plate. Pure cultures were obtained by subculturing individual colony on sterile PDA plates. PD broth (Himedia, India) was prepared, sterilized and inoculated with spores. Inoculated broths were maintained at 30 °C with moderate humidity in an incubator without shaking. A mycelial mat of 2-week-old culture weighing about 500 mg was harvested and washed with sterile double-distilled water. One gram lyophilized spore mycelial mat was crushed in liquid nitrogen and soaked in 5 ml sterile 0.1 M phosphate buffer (pH 7.2) for 72 h and centrifuged at $22,000 \times g$ for 20 min. The clear extract was obtained by filtering the supernatant through a 0.22- μm membrane (Millipore) and used for SPT. The protein content of the extracts was measured by using Bradford reagent (Bio-Rad Laboratories).

Skin prick tests were performed and wheal responses were measured after 20 min and graded (Stytis et al. 1982). Histamine diphosphate (1 mg/ml) and phosphate buffer (0.01 M, pH 7.2) were used as positive and negative controls, respectively.

2.9 Statistical analysis

The relationship between atmospheric fungal spore count and meteorological variables was studied by Spearman's rank correlation test (two tailed). 't' test was performed to compare the total and individual spore concentration in both the study years. Correlations between the total number of patients, total spore concentration and spore richness in each month of both the years were tested by Spearman's rank correlation analysis. One-way ANOVA followed by Tukey's multiple comparison tests was performed to compare the numbers of patients of different age groups. SPT responses to different fungal antigenic extracts in both male and female patients are tested

through paired *t* test. Statistical analysis was performed using Prism version 6 (Graph Pad Prism, San Diego, California, USA).

3 Results

3.1 Quantitative analysis of the aerial fungal spectrum

From the 2-year-long study, a total of 38 fungal genera were identified. The total aerospora concentration fluctuated between 26705.611 spores/ m^3 in the first year (2012–2013) and 27,910.912 spores/ m^3 in the second year (2013–2014). Among the aerospora recorded, the predominant ones (mean annual contribution > 1.0%) were as follows: *Bispora*, unidentified ascospores, unidentified basidiospores, *Ganoderma*, *Nigrospora*, *Pithomyces*, *Drechslera*, *Cladosporium*, *Alternaria*, *Aspergilli*/Penicilli, *Curvularia*, *Fusarium*, *Periconia*, rust spores, *Trichoconis*, etc. (Supplementary Table 1). Statistically significant differences were observed between the 2-year aerial concentration of *Cladosporium*, *Curvularia*, *Ganoderma*, *Pleospora*, *Podospora*, *Spegazzinia* and *Xylariaceae* ascospores when the consistency in spore concentration was checked by Student's *t*-test (paired). However, the remaining aerospora, as well as the total aeromycota, exhibited no statistically significant differences in the spore concentrations recorded in the two consecutive sampling years (Supplementary Table 1).

August was found to be the most spore-rich month (37 and 32 spore types identified, respectively, for both study years), and maximum spore concentration (7499.635 spores/ m^3 in the first year and 6914.102 spores/ m^3 in the second year) was found in this month as well. In both the years, the least spore-rich months were December and January with 14 and 15 spore types, respectively. Throughout the winter season (December and January), spore concentration also remained less and reached the least in the month of March (Supplementary Table 2), and this fluctuation pattern was indifferent to two consecutive years. Positive correlation was found between (1) the patients' visit to the clinic and monthly spore count, (2) monthly spore count and spore richness and (3) patients' visit to the clinic and spore richness. The significant *p* values for correlation (*p*) and regression

co-efficient values (R^2) are indicated along with the linear regression graph (Fig. 4). The R^2 values indicate the level of association between the variables in each case (Fig. 4a–f).

For the first time in a typical urban area of Kolkata, fungal spore calendar with consecutive 2-year records has been prepared (Fig. 1). In the calendar, it has been demonstrated that some spores were seasonal (e.g., *Coprinus*, *Epicoccum*), some spores were sporadic (*Cercospora*, *Tetracoccosporium*), and some spores were present throughout the year (e.g., *Curvularia*, *Ganoderma*).

The average result of the 2-year survey showed different patterns of intradiurnal periodicities for various spore types (Gregory 1973). Some showed maximum concentration at a particular time of the day, and some exhibited more than one maxima either during the daytime or night. The patterns were

categorized into night pattern (18.01–05.00 I.S.T.), post-dawn pattern (5.01–10.00 I.S.T.), middle day pattern (10.01–18.00 I.S.T.) and double maxima. *Ganoderma*, *Nigrospora*, Ascospores, and Basidiospores fall under night pattern category. Rust spores, *Periconia*, *Fusarium* spore concentration reached a peak in the early morning/morning times, and these are grouped into the post-dawn pattern. Spores showing forenoon (*Bispora*), noon (*Curvularia*) and afternoon peak (*Cladosporium*) fall under the middle day pattern. Lastly, Aspergilli/Penicilli exhibiting more than one peak within a day was grouped into double maxima category along with *Trichoconis* that showed the same pattern in the night time (Supplementary Fig. 1).

When Spearman's rank correlation analysis was performed to find any relationship between the meteorological factors with total spore concentration,

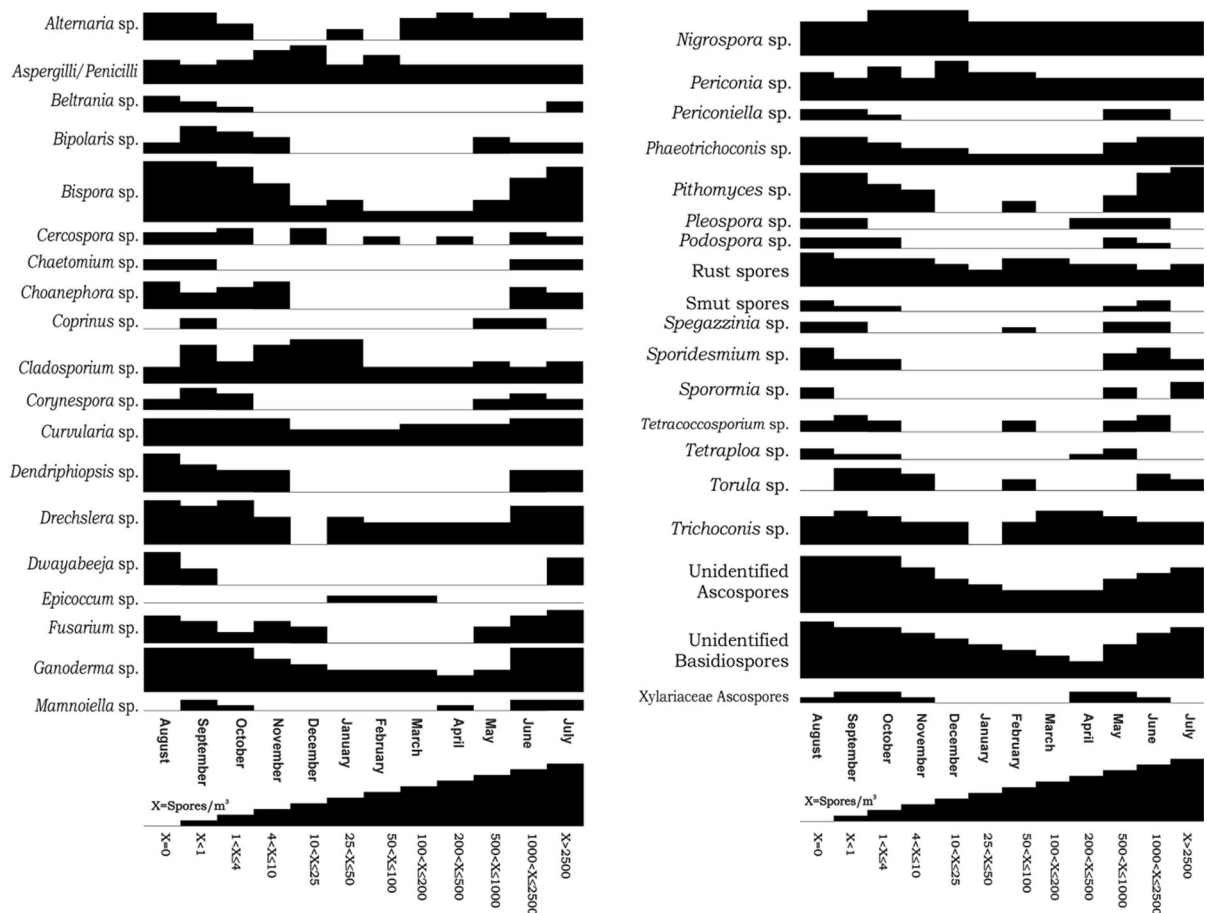


Fig. 1 Fungal spore calendar of Kolkata (August 2012–July 2014) representing the seasonal periodicity patterns of 38 aerospora in average values for a 2-year sampling period. The scale with the concentration levels of fungal spores is presented

we found that the total aeromycota have a significant positive association with relative humidity in the first year and relative humidity along with rainfall found to exert a significant effect in the second year (Supplementary Table 3). For individual fungal genus, *Pithomyces*, *Trichoconis*, *Periconia*, *Ganoderma*, *Aspergilli*/Penicilli and *Curvularia* spore concentration was found to be regulated by more than one climatic factor (Supplementary Table 4), whereas climatic factors did not have such impact on *Fusarium*, rust spores, *Nigrospora* and *Cladosporium* concentration.

3.2 Qualitative analysis through clinical investigation

Skin prick test was performed with 20 fungal extracts, and this selection was based on their aerial dominance and availability. Among 742 patients tested, 592 elicited $> +1$ SPT reactivity toward one or more fungal extracts. Among the fungal genera tested, maximum sensitization was shown by different species of *Aspergillus*. *A. fumigatus*, *A. flavus*, *A. versicolor* and *A. niger* collectively evoked 15–49% reactivity and hence appeared as the most sensitizing genus of that area. When we looked into the sensitization potential of individual fungal extract, highest reactivity was shown by *Aspergillus fumigatus* (49.1%) followed by *Curvularia pallescens* (38.8%) and *Fusarium lateritium* (31.9%) by patients when performing SPT. Other significantly reactive species were *Rhizopus oryzae* (13.7%), *Curvularia lunata* (11.8%), *Alternaria tenuis* (11.8%), *Cladosporium cladosporioides* (11.8%), *Fusarium solani* (10.0%) and *Neurospora sitophila* (10.5%) (Fig. 2).

When patients' visit to the allergy clinic was investigated in each month of both study years, maximum numbers of patients were found for the duration of August to October in both the years. Age group-wise categorization of the patients revealed that 15–35-year age group suffered most from fungal allergy (Fig. 3a). One-way ANOVA test showed that differences in the age group of affected patients are statistically significant ($F = 10.35$, $p < 0.0001^{***}$). Tukey's multiple comparison tests revealed enhanced number of SPT-reactive patients of 15–35 years of the age group in general (Fig. 3b). It was also found that male patients were more prone than females in general (Fig. 3c). The t-test revealed that a total number of

male and female patients eliciting positive SPT response differ significantly as well ($t = 3.616$, $p < 0.0018^{**}$; Fig. 3d).

Major symptoms noted in the patients were bronchial asthma, allergic rhinitis, urticaria, eye problems and so on (Table 1). Patients showed the above said symptoms singly or in a combination in most of the cases, and bronchial asthma was the most frequent symptom reported in a maximum number of patients (40.02%).

Patients having a family history of doctor-diagnosed allergic symptoms (63.2%) showed either sudden or insidious onset of allergic symptoms, whereas patients whose any of the family members have never encountered any allergic symptoms experienced insidious symptoms only (Table 1). This kind of insidious onset was associated with a change in living place or change in occupation that led to an increased exposure level.

4 Discussions

Many studies have been executed to find an alliance between airborne fungal spore exposure and the onset of allergic disease, but in many cases a direct cause–effect link has not yet been established correctly (Babu et al. 1979; Roby and Sneller 1979; Bavbek et al. 2006; Taylor et al. 2006). Improved knowledge of the epidemiology and mechanisms of fungal-induced human disease will hopefully unveil this causal link and suggest methods to lessen allergy-related health hazards. The present study was the first of its kind to be performed in a typical urban area of Kolkata, India.

We found almost equal total annual spore load in our two study years. The prevailing climatic conditions of India favor the growth and sporulation of fungi. As a result, airborne concentration of fungal spores tends to be high and nearly indifferent in two consecutive years.

The spore calendar indicating the monthly quantitative occurrence of airborne fungal spores and intradiurnal periodicity pattern showing peak time of occurrence of a particular fungal genus would be beneficial for any professional allergologist. The seasonal and intradiurnal variation of various aerospora observed in the present study were found to be similar with previous reports from a suburban area (Das and Gupta-Bhattacharya 2008). Intradiurnal

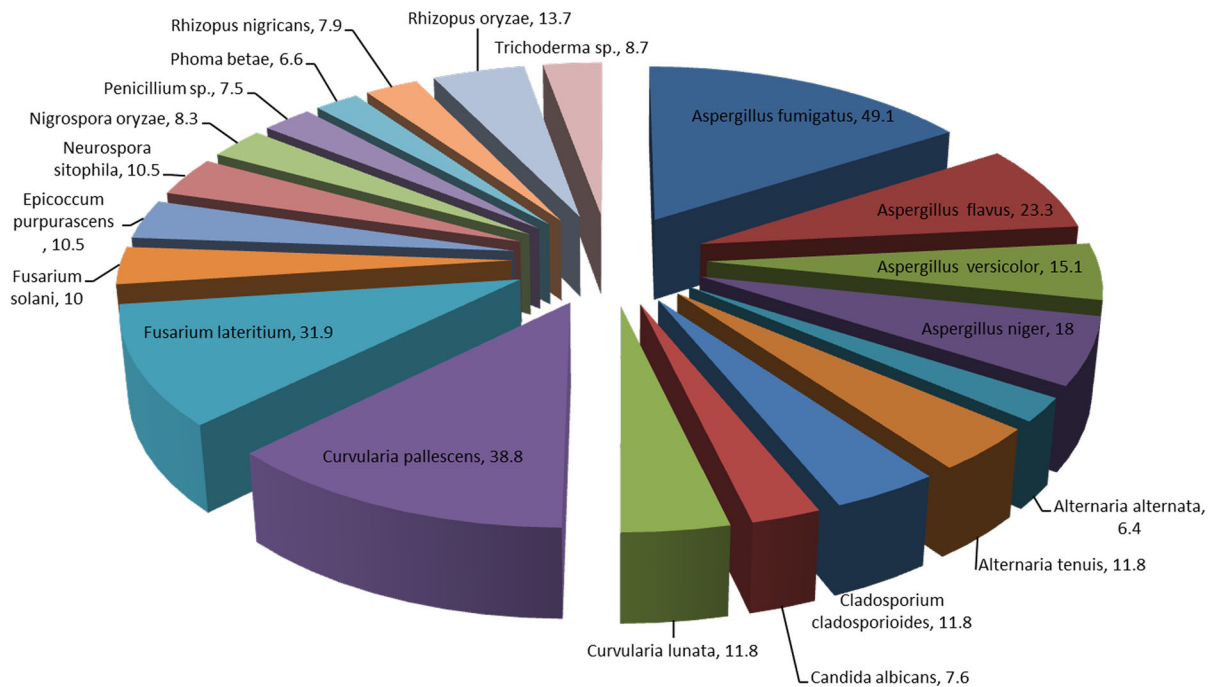
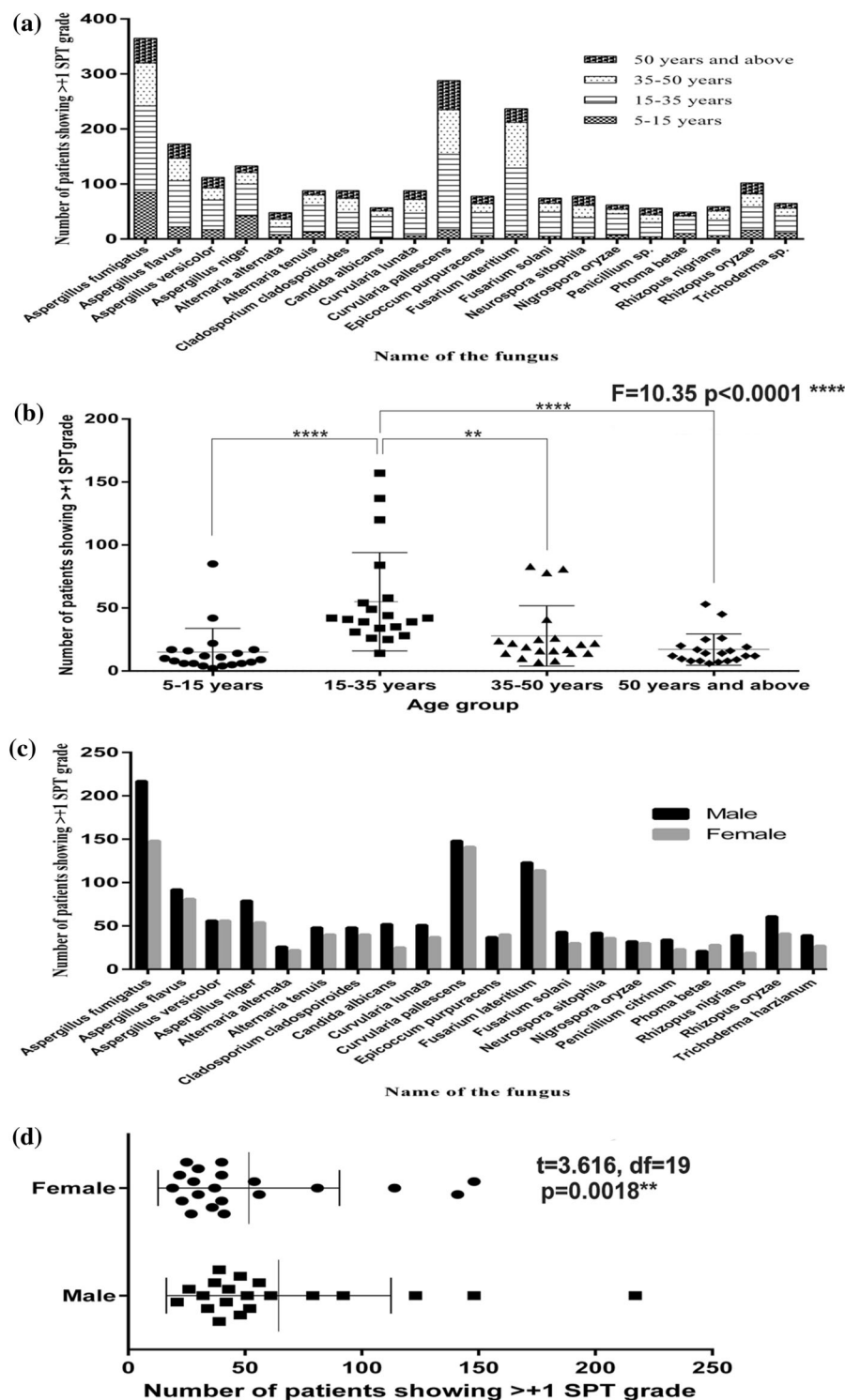


Fig. 2 Percentage of patients showing positive ($> + 1$) SPT grade with antigenic extracts of dominant spore types

periodicity pattern of some dominant fungal spores deviated significantly from a previous study of the fungal spores of the same suburban area (Chakraborty et al. 2003). Except for ascospores, basidiospores, *Cladosporium* and *Aspergilli*, all other fungal genus showed a more or less deviation in their intradiurnal periodicity peak pattern. Spore calendar revealed some variations from ours, and the reason can be explained by the differences in exposure between a suburban and a typical urban area. *Aspergilli*/*Penicilli*, *Cladosporium*, *Curvularia*, *Ganoderma*, *Nigrospora*, *Periconia*, unidentified ascospores and basidiospores are found throughout the year in both the calendar. Aerial concentration of *Alternaria*, *Chaetomium*, *Drechslera* and *Pithomyces* showed a seasonal fluctuation in our study, whereas these were found to be consistent throughout the year in a previous report (Das and Gupta-Bhattacharya 2008). Local environmental variables, edaphic factors, availability of fungal growth substratum as well as vegetation surrounding the trapping site played a significant role regarding this (Oliveira et al. 2009). Also, high levels of vehicle emissions may have an impact on the aerial components of urban areas.

The active release of fungal spores depends on changes in moisture conditions. Some fungal spores are released as the spore-bearing cell absorbs water, either during rainfall or as humidity increases. During long gentle rains, release mechanisms strongly exceed washout of spores by rainwater, often leading to much higher spore concentrations during rain. Most of the average annual rainfall of 175 cm, about 125 cm occurs during the rainy season (June to September) in Kolkata. This is probably the reason for obtaining the highest spore concentration in the rainy seasons for both the study years. This finding is in agreement with some earlier reports (Grinn-Gofron 2008; Lim et al. 2009; Stepalska and Wolek 2009). Some previous studies exhibited such kind of positive correlations between total spore concentration and relative humidity (Chakraborty et al. 2003). A study on Sivrihisar (Eskisehir), Turkey, revealed a positive correlation between relative humidity and spore concentration (Erkara et al. 2009). Another investigation on the fungal spores in the atmosphere of Rzeszów (southeast Poland) revealed the lowest spore concentration in winter that we found too (Kasprzyk et al. 2004).

Fig. 3 **a** Age group-wise categorization of patients for SPT response with different fungal extracts. **b** Statistical analysis to compare age groups through one-way ANOVA followed by Tukey's multiple comparison test (**significance level 0.01 and ****significance level 0.0001). **c** Gender-wise distribution of patients for SPT response with different fungal extracts (**d**). Results of paired *t*-test for the comparison of SPT reactivity between male and female patients



From the statistical analysis, it can be inferred that increased spore concentration is directly correlated with the rise in allergy patients' hospital visit (Fig. 4a,

d). Positive correlation also found between spore richness with spore concentration (Fig. 4b, e) and patients' visit to the hospital (Fig. 4c, f).

Table 1 Demographic and clinical history of patients

Demographical and clinical features	Total number of patients	% of patients
<i>Age groups</i>		
< 15 years	103	13.9
15–35 years	220	29.6
35–50 years	210	28.3
> 50 years	209	28.2
<i>Gender</i>		
Males	398	53.6
Females	344	46.4
<i>Symptoms</i>		
Allergic rhinitis	214	28.84
Bronchial asthma	297	40.02
Urticaria	216	29.11
Eye problem	177	23.85
Other respiratory discomforts	121	16.3
<i>Family history of atopic diseases</i>		
Yes	469	63.2
No	273	36.8
<i>Nature of onset of symptoms</i>		
Sudden	174	23.45
Insidious	568	76.55

Large spores ($> 10 \mu\text{m}$ in size), deposited usually in the nasopharynx, are associated with nasal and ocular symptoms, resulting in allergic rhinitis, whereas smaller spores ($< 10 \mu\text{m}$; specially $< 5 \mu\text{m}$ in size) can penetrate the lower airways and trigger bronchial asthma (Luo 1991). We found both bronchial asthma and allergic rhinitis as prevalent symptoms in the investigated population. These symptoms were found to be prevalent in other studies performed in Kolkata before (Das and Gupta-Bhattacharya 2008; Chakrabarti et al. 2012). But urticaria and eye problems were not previously reported to be severe in nearby populations before.

As our study was based on the outdoor environment only, it can be inferred that young populations are more likely to have exposure of the outdoors for a longer time in a day and this reason authenticated our finding of the maximum suffering of patients of 15–35-year age group. Again male patients showed more SPT reactivity than females in most of the fungal extracts. This phenomenon can be explained by considering the fact that an adult male inhales

10,000 L of air daily, so the inhalation of airborne agents can have an elevated effect in males and this finding was supported by earlier researchers (Chakrabarti et al. 2012).

However, development of civilization often at the expense of the natural environment by pollution stimulates the emergence of new health hazards; allergic diseases are one of those. We found that sudden onset of allergic manifestations was due to change in living place or occupation, which ultimately led to a change in allergen exposure level and subsequent onset of symptoms.

Considering the qualitative and quantitative investigations together, it is found that *A. fumigatus*, *C. pallescens*, *F. lateritium* elicited maximum skin sensitizing potential and were also dominant in their aerial concentration of the study area. Thus, these particular fungal species seem to pose a potential threat to the local atopic populace and are significant for further consideration for identification and characterization of allergens leading the way to immunotherapy.

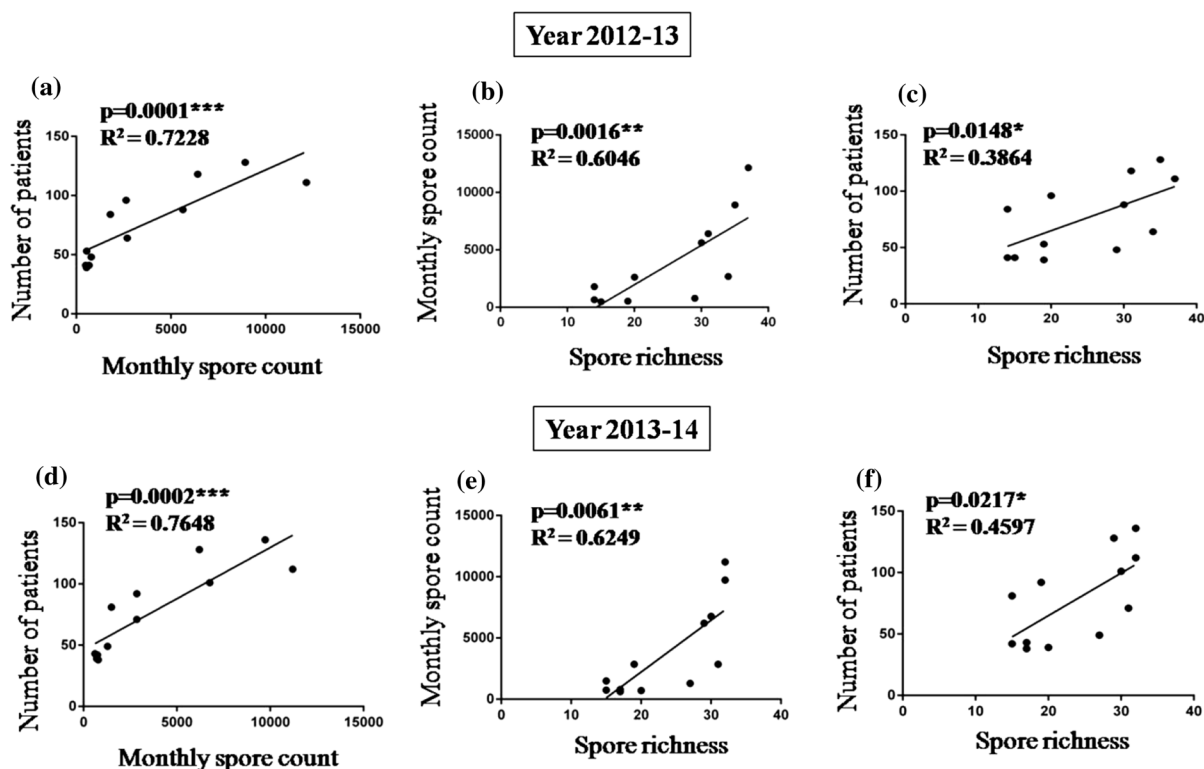


Fig. 4 Correlation and linear regression analysis of (i) monthly patients visit to the clinic and monthly spore count (a, d), (ii) monthly spore count and spore richness (b, e), (iii) monthly patients visit to the clinic and spore richness for two study years (c, f)

5 Conclusions

The present study provides detailed information on the aeromycoflora content and their impact on the local populace. This kind of investigation was done for the first time in a typical urban area of Kolkata. Irrespective of the type of allergic symptom, allergies are chronic disease severely affecting the quality of life and may even sometimes be fatal. Because the presence and abundance of outdoor allergens cannot be managed, avoidance of outdoor allergen exposure is supposed to be the key strategy for allergy treatment. Proper treatment may require a change in lifestyle or profession maintaining allergen avoidance, long-term pharmacotherapy and even immunotherapy.

Acknowledgements This work is supported financially by University Grants Commission (Grant No. F.2-8/2002(SA-I)), Government of India. Authors are thankful to the Director, Bose Institute, Kolkata, for providing infrastructural facilities. The authors wish to express sincere gratitude to the medical professionals and clinical staffs of Mediland Diagnostics,

Kolkata, for executing the clinical aspects. Sincere thanks to Chanchal Chakraborty, Soumya Subhra Gupta, Jadab Kumar Ghosh, Kaberi Ghosh and Asish Bera for technical help.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the clinic.

Informed consent For this type of retrospective study, formal consent is not required.

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