3.1. Sampling site

Chennai is the fourth most populous metropolitan area and the fifth most popular city situated at Latitude 13° 04’ N and Longitude 86° 17’ E on the Southeast coast of India. Chennai metropolitan area extends over 1180 sq km and has a population of more than 8.5 million. Rapid urbanisation with vehicle congestion has increased menacingly on the roads of Chennai. As a result of this, gaseous pollutants and respirable suspended particulate matter pollutants are continuously increasing in the ambient air, consequently an increase in the incidence of respiratory allergic diseases are being reported in Chennai.

3.2. Survey protocol

The present study measured the bacterial and fungal concentrations in the outdoor and indoor air of various microenvironments under uncontrolled environmental conditions from January 2007 to December 2008. Samples from each microenvironment were surveyed for bacteria and fungi throughout the study period. An effort was made to geographically disperse the sampling sites throughout the city. It was expected that the summer would provide a more favourable humid environment for microbial growth, resulting in different bioaerosols levels. The indoor and outdoor air measurements were taken concurrently or consequently at each sampling site. The majority of the indoor air measurements were taken from the middle of the facility or living area at breathing height, while the outdoor air measurements were taken from outside area of the surveyed facility.

The microenvironments selected from outdoor were Bus stand, Railway station, Recreation ground, Sewage treatment plant and Vegetable market and indoor such as Home, Hotel, Office premise, Public toilet and Theatre.

3.3. Schedule of air sampling

The air sampling was performed during regular morning (between 10am – 12 noon) and regular evening hours (between 17:00h and 19:00h) on week days (Monday to Friday). Homes selected from typical residential areas were surveyed and each residence was occupied by a single family with three to six persons. All the households participated
the day. For comparison purposes, measurements were also made, the periods of direct sunlight were minimal because of the closed environment was recorded. This increases the probability of survival of air borne microorganisms as the sun’s direct UV radiation can reduce the viability of many microorganisms. However, the microenvironment of theatre samples was collected from the rear of the theatre to minimize the interruption, and the sampling was performed during the show time and intermission times to examine bioaerosols levels. The same survey was also performed in public toilets.

A total of 10 microenvironments (5 from each environment) were selected for the study. From each microenvironment, 12 samples were surveyed at regular intervals of a month for the study period. Each sample was a mean of triplicate, which was collected once in 10 days intervals.

3.4. Air sampling methods

3.4.1. Sampling device

The sampling device, the Air Petri Sampling System Mark II (Hi media laboratories limited, India) is highly specialized instrument devised to collect and to enumerate microorganisms by aerodynamic fractions (Archana, 2004) based upon the principle of sieve impactor as described by Anderson (1958) which aspirates air through a perforated plate. The instrument consists of a powder coated aluminium container with clamp designed to accommodate a petridish containing a nutrient agar or any other desired medium. The unit consists of powder coated tall stand, adaptor for mounting (either vertical or horizontal direction), SS cone (sampling system) for 90 mm petriplate and SS feeder cone circular clamp with SS feeder cover. The impactor stage contains 340 precision drilled holes. When air is drawn, the air borne particles are impinged towards the surface of the agar. The diameter of the orifice is 1.5millimeter. The schematic picture of a sampler as a whole is shown in figure 1 and the sampler is operated at the flow rate of 100 L/min. The portable sampler is operated by a remote system using chargeable batteries.
3.4.2. Preparation of the sampler

Sampler fractionating units were sterilized in hot air oven at 160°C for 1 h before using. Prior to the sampling process the fractionating units were swabbed with cotton and dipped in 70% ethyl alcohol and samplings were taken only after the evaporation of alcohol. The autoclaved SS cone was fixed in the sanitized sampler; petriplate of 90 mm diameter was kept in the sampler with 20 ml of culture medium which serves as collection surface. The plate resets on the raised metal pins. Lid of the petriplate was removed and immediately the plate was covered with autoclaved SS feeder cone circular clamp assembly in position. The device was visually checked to ensure a good sealing.

3.4.3. Air sampling

The sampler was placed centrally within each microenvironment and was raised to a sampling height of approximately 1 meter. The agar plate with its lid removed was placed on the base of the sampler, so that the plate rests on raised metal pins. Immediately the plate was covered with the SS feeder cone with SS feeder cover and the device was secured tight and
visually checked for good seal. The cover of the unit is perforated, with a perforation of
predetermined size. A vacuum pump draws a known volume of air through the SS feeder
cone with where it is accelerated and passes onto the stage, and the particles in the air
containing microorganisms impinge on the agar medium in the petridish. The exhaust air is
then carried through the outlet on the base and relaxed outside.

After sampling, the inlet cone was released and the agar plate was removed. The agar
plate cover was replaced quickly and labelled at the back with the appropriate sampling and
identification information. The sampler was resanitized with 70% ethanol wipe between each
sample location and allowed to dry. All exposed plates were transported to the laboratory for
incubation.

Nutrient agar, Blood agar, MacConkey agar and Potato dextrose agar (Appendix I)
were used for the enumeration, isolation, and propagation of different microbial communities.
The exposed petriplates containing media were sealed with parafilm and transported to the
laboratory for incubation. The plates were incubated at 37° C for 24 – 48 h for bacterial
growth and at room temperature (28 ± 2° C) for 48 – 72 h for the isolation of mesophilic
fungi. The counts for the air sample plates were corrected for multiple impactions using the
positive hole conversion method (Anderson, 1958) and reported as colony forming units per
cubic meter (cfu/m³) of air. The colonies were then identified and subcultured for further
studies.

3.5. Identification of bacteria

The isolated bacteria were identified through microscopic examination, biochemical
testing using Cowan and Steel’s manual for the identification of bacteria (Cowan, 1974).

3.5.1. Preliminary tests

3.5.1.1. Gram staining

The organism on culture was stained using Gram staining method. The smear
preparation, staining and fixation, decolourization and counter staining were carried out
conventionally and observed under the microscope for bacteria.

3.5.1.2. Motility test

Clean cavity slides along with cover slips were taken for each isolate. A drop of
culture suspension was placed at the centre of the cover slip and vaseline was applied to the
corners of the cover slip, which was then placed over the cavity slide. The slide was then viewed microscopically to check the motile nature of isolates under 40X.

3.5.1.3. Catalase test – slide method

Pure culture of the isolate was transferred from the agar to a clean slide with a loop or glass rod. Immediately, a drop of 3% hydrogen peroxide was added to the culture and observed for effervescence. Negative and positive controls were kept for confirmation.

3. 5. 1.4. Oxidase test

Oxidase enzyme plays a vital role in the operation of electron transport system. This test depends on the presence of certain oxidases in bacteria that catalyse the transport of electrons between electron donors in bacteria and a redox dye tetramethyl – p – phenylenediamine dihydrochloride. The dye is reduced to a deep purple colour. Oxidase disc was placed on a clean glass slide which was then placed in the petridish. The dish was moistened with distilled water. The colony to be tested was picked up using a tooth pick and smeared over moist area. Then the colour development was observed.

3.5.2. Biochemical tests

3.5.2.1. Indole production test

The ability to hydrolyze tryptophan with the production of indole is detectable by adding Kovac’s reagent composed of p – dimethyl aminobenzaldehyde which produces a cherry red colour. Sterile peptone broth, 5 ml was inoculated with the culture and incubated at 37° C for 48 h. Following incubation, 0.2 ml of kovac’s reagent was added to observe the colour change.

3.5.2.2. Methyl red test

Sterile glucose broth, 5 ml was inoculated with the test culture and incubated at 37° C for 48 h. Following incubation, 5 to 6 drops of methyl red solution was added to detect the ability of microorganisms to oxidize glucose with the production and stabilization of high concentrations of acid end products.

3.5.2.3. Voges – proskauer test

This test is to determine the capacity of some organisms to ferment carbohydrates with the production of non-acidic or neutral end products. Sterile glucose broth, 5 ml was inoculated with the test culture and incubated at 37° C for 48 h. Following incubation 1ml of
40% potassium hydroxide and 3 ml of 5% solution of alpha naphthol in absolute ethanol were added to observe the colour change.

3.5.2.4. Citrate utilization test

Some microorganisms use citrate as the sole carbon source and grow. Citrate is acted upon by the enzyme citrase which produces oxaloacetic acid and acetate. These are then enzymatically converted to form products. This reaction is shown by the change in the colour of the indicator. The medium was streaked with broth culture of test organism and incubated at 37° C for 24 h. After incubation period, colour change was noted.

3.5.2.5. Triple Sugar Iron (TSI) agar test

TSI test is used to differentiate different groups of Enterobacteriaceae according to their ability to ferment lactose, sucrose and glucose and the production of hydrogen sulphide. The fermentation reaction of the sugars will help to distinguish organisms. The TSI slants contained 1% each of lactose, sucrose and glucose in a concentration of 0.1%. The phenol red, the acid base indicator was incorporated in the medium to detect carbohydrate fermentation. The medium was inoculated with the test culture by first stabbing the butt down to the bottom and then streaking the surface of the slant and incubated at 37° C for 24 h to observe the colour change.

3.5.2.6. Urease test

Urease is an enzyme produced by few microorganisms. Urease is a hydrolytic enzyme that attacks the nitrogen and carbon bond in amide compounds like urea and forms alkaline end products such as ammonia. Production of urease is detectable when the organisms are grown in urea broth medium containing phenol red, pH indicator which shows the change in colour. This medium was inoculated with the test culture and incubated for 24 h at 37° C and the colour change was noted.

3.5.2.7. Carbohydrate fermentation test

Most microorganisms obtain their energy through a series of orderly and integrated enzymatic reaction leading to the bio - oxidation of a substrate, frequently a carbohydrate. In fermentation, substrates such as carbohydrates and alcohols undergo anaerobic dissimilation and produce an organic acid that may be accompanied by gases such as hydrogen or carbon
dioxide. The broth was inoculated with the test culture and incubated at 37° C for 24 h and observed for gas production.

3.5.2.8. Oxidative fermentation test

This method depends upon the use of a semisolid medium containing the carbohydrate (usually glucose) together with a pH indicator. If acid is produced only at the surface of the medium, where conditions are aerobic, and the attack on the sugar is oxidative. If acid is found throughout the tube, including the lower layers where conditions are anaerobic, the breakdown is fermentative. Duplicate tubes with medium were inoculated by stabbing. One tube was covered with liquid paraffin to a depth of 5 – 10 mm and both were incubated at 37° C for 48 h or longer and observed for acid production.

3.6. Identification of fungi

The exposed petriplates containing Potato Dextrose Agar (PDA) sealed with parafilm were transported to the laboratory for incubation. The plates were incubated at room temperature (28 ± 2° C) in the glass chamber for the isolation of mesophilic fungi. After incubating for a period of 2 to 3 days, the colonies were counted, identified and subcultured for further studies.

3.6.1. Morphological characteristics

3.6.1.1. Macroscopic appearance

Morphological characteristics of the culture viz, colour, shape, pigmentation, reverse pigmentation were studied by using the hand lens.

3.6.1.2. Microscopic appearance

Microscopic characteristics were studied by preparing the slides and observing under light microscope. The characters of conidia bearing structure, shape, size, separation, colour and ornamentation were observed. Lactophenol with cotton blue (for hyaline molds) was used for staining fungi. The prepared slides were sealed with DPX (Qualigens fine Chemicals) for preservation. The fungi were identified with the help of standard manuals and monographs (Gilman, 1957; Moubasher, 1993) which is based mainly on gross colony appearance and microscopic examination of the spore and mycelium.
3.7. Presentation of data

3.7.1 Air sampling (Kalogerakis et al., 2005)

The colonies of individual organisms were converted to number/m$^3$ of air by multiplying with a factor calculated as follows and the counts are expressed as colony forming units (cfu)/cubic meter of air (m$^3$).

Suction rate of the sampler = 100 L/min

Duration of each sampling = 4 min

Amount of air sampling in 4 minute = 4*100 = 400 L/m$^3$

Let the number of colonies recorded = X

Thus the number of colonies recorded /m$^3$ of air = 1000 /400* X

The conversion factor = 2.5

3.7.2. Isolation frequency

The term isolation frequency has been used to denote the number of sampling in which an organism was recorded as against the total number of samplings (in each microenvironment). On the basis of percent isolation frequency the organisms were grouped as,

Most common = above 80% and 100%

Common = 60% and below 80%

Frequent = 40% and below 60%

Occasional = 20% and below 40%

Sporadic 1% to below 20%

3.7.3. Percent contribution

The term percent contribution refers to the contribution of individual organism to the total and is calculated as follows

Percent contribution = No. of cfu/m$^3$ of an individual organism/ Total no. of cfu/m$^3$ of all organisms
3.8. Recording of meteorological parameters

Monthly mean temperature (°C), mean relative humidity (%), mean total rainfall (mm) and mean wind speed (km/h) were obtained from the Regional Meteorological centre, Government of India, Chennai. Geographic distribution, seasonal variation and annual trend of organisms were studied in detail.

3.9. Statistical analyses

The statistical analyses were performed using mini tab version and excel 2007 on a personal computer. Analysis of variance (ANOVA) was used for the comparison of data sets with monthly mean temperature (°C), mean humidity (%), mean rainfall (mm) and mean wind speed (km/h). Both Pearson’s correlation co – efficient and Spearman’s rank correlation co – efficient were calculated between different meteorological factors with aerosol concentration in each microenvironment during the sampling periods. A paired t – test was employed for the comparison of the bioaerosol data sets of the outdoor and indoor air. Meanwhile, a non parametric test (Mann – Whitney test) was employed for the comparison of the bioaerosol data sets of five different microenvironments each from outdoor (bus stand, railway station, recreation ground, sewage treatment plant and vegetable market) and indoor air (home, hotel, office premise, public toilet and theatre) and two sampling periods. The mean and standard deviation (SD) were used to characterise the normally distributed data. The criterion for significance in the procedure was P<0.05.

3.10. Immunological analysis

The present work investigated the indoor air quality in terms of fungal population and to evaluate between the microbial air quality and the allergic status of the selected individuals in the sampling site to study the immunoglobulin E antibody as the serological index to relate with the allergic status.

3.10. 1. Study design

Allergy is more common in urban areas when compared to rural areas. This clearly suggests that air population may play a possible role in allergic diseases. The relation between the microbial air quality and the allergic status of the selected individuals was evaluated by Enzyme Linked Immuno Sorbent Assay method. The most common invitro test for assay of allergen activity is ELISA (Wisdom, 1976; Turner et al., 1980).
This study was an incident case–control study. A total number of 115 blood samples were collected from the volunteers of the selected occupants with an age group of 21–64 years for the case study and a total of 25 samples were collected from the healthy individuals for control study living in the microenvironment (homes), a geographically defined industrialized and highly polluted area (Chennai). Recruitment of controls took place at regular intervals throughout the study period.

3.10.2. Selection of cases

Volunteers were selected from the microenvironment (homes), first in the city from January 2009 to August 2009, based on a questionnaire, to be used in general population, (Jaakkola and Miettinen, 1995; Jaakkola and Jaakkola, 1999) included: (i) health information (ii) active smoking (iii) occupation and work environment (iv) home environment and (v) dietary questions. As an additional route of case selection, a history of at least any one asthma symptoms such as, prolonged cough, wheezing or nocturnal cough was included for the study. All the confirmed cases of asthma like symptoms fulfilling the general eligibility criteria were selected for the case study. A total of 115 blood samples were collected for eosinophil counts, total IgE and specific IgE antibody analyses.

3.10.3. Selection of controls

The controls were drawn from the volunteers of the same microenvironment and the general criteria were also applied. Recruitment of controls took place at a regular interval throughout the study period. Blood samples were collected from 25 individuals (controls) and processed immediately.

3.10.4. Screening test

3.10.4.1 Differential WBC count (Parra et al., 2000)

Neutrophils, eosinophils, basophils, lymphocytes and monocytes are the five types of leucocytes normally found in blood. To determine the relative percentage of each type of leucocytes, the blood smear will be stained and examined under oil immersion. A total of 100 white blood cells will be recorded. This method of white blood cell enumeration is called a differential count. The blood smear was prepared and air dried. Then it was stained with Leishman’s stain and examined under oil immersion objective.
3.10.5. Estimation of total IgE

3.10.5.1. Enzyme Linked Immuno Sorbent Assay (ELISA) (Chowdary et al., 2003)

The collected blood samples were processed for the IgE level. Serum was separated by centrifugation and stored at -20° C before shifting to the laboratory for total IgE antibody analyses. The IgE quantitative ELISA provides a rapid, sensitive and reliable assay for total serum IgE. The minimal sensitivity of this assay is about 5.0 IU/ml. The individuals with atopic allergic diseases exhibit increased total immunoglobulin E (IgE) levels in blood. In general, elevated levels of IgE indicate an increased probability of an IgE – mediated hypersensitivity.

Materials and components

- Antibody coated microtitre wells, 96 wells per plate.
- Reference standards, 0, 10, 50, 100, 400 and 800 IU/ml (liquid).
- Zero buffer (13 ml)
- Enzyme conjugate reagent (18 ml)
- Substrate TMB (13 ml)
- HCl, 2N (10 ml)

Assay procedure

The entire reagents were brought to room temperature (18 - 25° C) before use. Lyophilized standards were reconstituted with 1.0 ml distilled water, and allowed to stand for at least 20 minutes. Reconstituted standards were stored at 2 - 8° C. The desired number of coated wells was secured in the holder. Standard specimens, 20 μl of each were dispensed into appropriate wells. Zero buffers (100 μl) was dispensed into each well and thoroughly mixed for 10 seconds. The plate was then incubated at room temperature (18 - 25° C) for 30 minutes. The incubation mixture was removed by flicking plate content into a waste container. The microtitre wells were washed with distilled water 5 times, with 30 seconds soaking time. The wells were then sharply tapped on an absorbent paper to remove all residual water droplets. Enzyme conjugate reagent (150 μl) was dispensed into each well and
gently mixed for 5 seconds. The plate was then incubated at room temperature for 30 minutes.

The wells were washed 5 times with distilled water with 30 seconds soaking time for each wash, after incubation. Tapping on an absorbent paper was done to remove the residual water droplets. TMB solution (200 μl) was dispensed into each well and gently mixed for 5 seconds. Incubation was carried out in the dark for 20 minutes. The reaction was stopped by adding 50 μl of 2N HCl to each well and gently mixed for 30 seconds (It is important to make sure that all the blue colour changes to yellow colour completely). Then optical density was read at 450nm with a microtitre reader. Finally the absorbance of the test serum was measured and compared with standards.

Values and sensitivity

Serum IgE values are expressed in International Units/ml. Laboratories working independently have confirmed that 1 IU equals approximately 2.4 ng of protein. The total IgE level in a normal, allergy free adult is < 160 IU/ml of serum. The minimum detectable concentration of IgE by this assay is estimated to be 5.0 IU/ml. To determine the IgE level of the patient, first the mean absorbance value for reference standards was established.

3.10.6. Estimation of specific IgE (Weir et al., 1987 and Brummund et al., 1987)

The estimation of specific IgE was done by Enzyme linked immunosorbent assay. In ELISA, antibodies that exhibit high binding affinities to different surface chemicals motifs of the immunogen are made use of. The method is based on the interaction of an antigen with its specific antibody.

- Coating buffer: The buffer (Carbonate – bicarbonate buffer, 100 mM, pH 9.6) was prepared by dissolving 840 mg of sodium bicarbonate and 1.06 g of sodium carbonate in 100 ml of distilled water. The pH of the buffer was adjusted to 9.6 with 0.1N HCl

- Blocking buffer: Phosphate Buffer Saline (PBS), 10 mM, pH 7.2 with 0.1% fish gelatin and 0.05% (w/v) sodium azide.

- Washing Buffer: Phosphate buffered saline - Tween (PBS – T), 0.01 M, pH 7.2 containing 0.05% (v/v) Tween – 20 and 0.01% sodium azide.
Stopping reagent: NaOH, 5N was added as a stopping reagent and it was prepared by dissolving 20 g of NaOH in 100 ml of distilled water.

Allergen extracts: Allergen to be coated on the microtitre plate was prepared by mixing 38.5 μl of allergen extract with 11.5 μl of coating buffer to get an antigen concentration of 10 μg/50 μl of buffer.

Few wells of microtitre plate were coated with 50 μl of allergen extract prepared (50 μl/well) in coating buffer. Another set of few wells were coated with coating buffer containing fish gelatine, which serves as blank. Plates were dried overnight at 37° C in an ELISA incubator. After incubation, antigen coated plates were washed six times with washing buffer. Wells were blocked for non specific binding with 100 μl blocking buffer/well and incubated at 37° C for 30 minutes. Plates were washed six times with washing buffer and 50 μl of patient’s serum/well (sera showed positivity for total IgE) was added and incubated at 37° C for three hours. Plates were then washed eight times and 150 μl of substrate buffer was added to each well, kept for incubation at 37° C for 45 minutes. Finally, 100 μl of stopping reagent (5N NaOH)/ well was added. The absorbance was read at 405 nm.

3.11. Air sanitation

Test standard

- No standard method for evaluating air sanitizers has been adopted. Referring to the attached references for information on testing products intended for sanitizing the air of enclosed spaces.
- The quantitative microbiological assays must be performed, using an air sampling device, to show the level of reduction of viable microorganisms achieved with the product, used as directed, in an enclosed experimental room or chamber (Wolf et al., 1959).
- The methodology employed, such as spraying and sampling procedures, and the environmental conditions in the room or chamber, such as temperature, relative humidity, etc., must be reported. Interpretation of the results must be included in the reports.
The results must show the adequate vapour concentrations are achieved in the air of the test enclosure. The results must also show a viable count reduction at least 99.9% over the parallel untreated control, after correcting for settling rates, in the air of the test enclosure.

A mouldy home, the risk of asthma for its residents, associated with an exposure to the total mould or to some specific genera, probably increases with the inhalation of fungal particles as well as their products. Hence, proper identification and elimination of the microbial source in occupational and house hold settings, use of filters in ventilation and air cleaning by the use of disinfectants and biocides. Bacillocid (Ghosh et al., 2004) is the commonly used, commercially available surface and environmental disinfect that has very good cleansing property along with bactericidal, fungicidal, viricidal and sporicidal activities. It does not require shutdown of the contaminated areas for 24 h.

**Application**

The application was made in closed spaces (all doors and windows were closed; air conditioners were turned off). The prepared solution (0.5% Bacillocid) was sprayed in wash floors, other tiled surfaces taking care to cover corners and other inaccessible areas by using soft sprayer in a closed room liberally allowing a contact time of 30 minutes and allowed to dry. The temperature and relative humidity were also recorded. Precautions were made not to ventilate rooms immediately after sanitation. Fans were switched off to slow down evaporation.

Sanitized High Air Sampling System Mark II was placed centrally within the sanitized room (home) and the agar plate (Potato dextrose agar) with its lid removed was fixed. The sampling was done after the device secured tight. After sampling the agar plate was removed and kept for incubation at ambient temperature (28±2°C) for 48 to 72 h. The sampling was done before and after sanitization to check its efficacy.

**Performance standard (Mc Gray, 1970)**

The results must show the adequate vapour concentrations are achieved in the air of the test enclosure. The results must also show a viable count reduction at least 99.9% over the parallel untreated control, after correcting for settling rates, in the air of the test enclosure.