MATERIALS AND METHODS


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TILAK
AIR SAMPLER
T MOHANRAO AURANGABAD M S

MATERIALS AND METHODS

Aerobiological sampling methods are very diverse and differ according to interest of individual and the components of airspora to be studied. In the earliest period, sample methods with help of kites or balloons were used which carry sticky coated slides along with them. These methods were useful to provide the qualitative data on the composition of airspora at different heights and exposure timing; however, today, these methods have become only of historical interests. These methods do not help in providing, a quantitative analysis and the composition of the airspora.

The various methods of sampling, have been summarized by Agarwal et al. (1973). Some of the methods used for air monitoring are Gravity sampler, Durham's pollen sampler, Individual pollen collector, Bourdillon slit sampler, Anderson's sampler, Impaction sampler, Rotorod sampler, Gravity Petridish method, Filtration method etc. These methods are used as per the purpose and utility.

Hospital, is an important indoor environment responsible for transmitting potential pathogenic or allergenic microbes to the susceptible persons. Environmental bioallergens like fungal spores and pollen grains may initiate allergic response to the susceptible individuals. Allergic people have capacity to react to potential allergens. It may be hypersensitive to them causing several types of eye, skin or respiratory disorders. Thus, hospital environment is responsible both for indoor and outdoor community. Various groups of people visit the hospital daily for different purposes viz. doctors, nurses, patients, sweepers, relatives, visitors etc., every day for the treatment and other purposes. Some of them certainly sick by skin and other diseases viz. apergillosis, blastomycosis, candidiasis, cladosporiosis, mucormycosis, mycotic keratitis, penicillosis, mycetoma or other mycoses. The spores or conidia occur in the indoor and around the hospital. Keeping this view in mind, aeromycological survey was taken, continuously from October 2006- September 2007. To study the hospital airspora and % of the patients, Spot sampling method was used. Two methods were adopted in this investigation as-

1) By using Tilak Rotorod sampler method (Tilak 1967, Tilak et al.1972).

2) Gravity petridish method.

1) Tilak Rotorod sampler method- The rotorod sampler has been used for a wide variety of airborne particles. Dr. W.A. Perkins (1957), developed a battery operated rotorod sampler. The device relies upon the high efficiency with which small air

borne particles are deposited on narrow cylinders, oriented at right angles to high velocity winds. A small, constant speed battery operated motor is used to whirl, thin sticky coated brass rods about its axis at a constant speed. It is a cheap, portable and high efficiency sampler with high sensitivity. Collecting arms of the model are made up of 0.159cm (1/16 inch) square. The section brass rods are slightly bent inwards. The vertical arms are 6 cm long and 4 cm away from the axis. The model employses D.C. controlled speed motor. The volume of air swept, can be calculated from the frontal area of the rod, the diameter through which, it is turned and the number of revolutions for which it is run. In the present investigation, Tilak rotorod sampler was used.

Sampling rate- The sampling rate is the volume of air swept over by the collecting surface per unit time. The volume of air can be calculated on the basis of the dimensions.

$= 2(\text{arms}) \times 0.159\text{cm} \times 6\text{cm} \times 8 \times 2300 \times 10^{-3}$

$= 48.0 \times 10^{-3} \times 2300$ liters/ min.

= approximately 100 liters / min.

Sampling surface- The sampler was originally intended for direct observation of spores, pollens, insect parts, etc. There is no necessity of mounting. Adhesive tape was fixed around the two arms of air sampler and sampler was allowed to run. The edges of the cellotape are trimmed back to the width of the rods with a sharp razor blade. The cellotape is cut into four equal parts (each 1.5cm length) and mounted on a glass slide. Tape was fixed on the slide by its sticky face and observed directly under the microscope at various magnifications. Different types of spores, conidia, basidiospores, ascospores, teleutospores, blastospores are generally observed on the basis of microscopic characters [Plate fig. nos. I and II by Tilak rotorod Sampler method].

Sampling site- Rotorod sampler was kept inside the general wards of the Krantisinh Nana Patil, General Hospital, Satara at a height of 1 meter from the ground level for about an hour.

2) Gravity petridish method- Gravity petridish method was also applied to coincide the results of rotorod air sampler method. Petridishes with sterile fungal media were exposed to grow the fungi and bacteria. For this purpose PDA medium was used.

The composition of the PDA medium is as follows-

Potato (peeled)	200.0 g
Dextrose	20.0 g
Agar-Agar	15.0g
Distilled water	1000.0 ml

The sterile culture plates were exposed inside and outside of the hospital for 15-20 min. twice in a week.

Laboratory Observations - The petridishes were brought in the laboratory. These exposed petri-plates were incubated for various periods, by keeping them in inverted position. The colonies were properly observed at an interval of 4, 8 and 15 days and were counted on colony counter. The density, color, dimensions, thickness, margin, edge, texture etc. i.e. the primary observations were taken.

Mycological Stains- The mycological stains are used to stain different fungal structures such as mycelium, asci, ascospores, conidia, conidiophores, pseudoparaphyses etc. In the present investigation cotton blue in lactophenol was used to study different fungal structures.

Cotton blue - It is an acidic stain used to study fungal structures. It is a cytoplasmic stain. The cyptoplasm turns blue in color, leaving the hyphal or spore wall and the septa, hyaline. Therefore, the size, shape, septation, structure of the ascospores, spores, conidia and mycelium can be studied.

Components used in preparation of cotton blue in lactophenol –

Lactic acid	20.0 ml
Phenol crystals	20.0 g
Glycerol	40.0 ml
Distilled Water	20.0 ml
Cotton blue (1% aqueous)	2.0 ml

Lactophenol – It is used as a mounting medium. Semi-permanent slides were mounted in lactophenol.

Components of lactophenol are as follows-

Lactic acid	20.0ml
Phenol crystals	20.0 g
Glycerol	40.0ml
Distilled Water	20.0ml

The fungal materials were stained with 1 % cotton blue and mounted in lactophenol and were sealed with DPX or wax sealant. The microscopic observations viz. mycelium, hyphae, color, spore, septations, fructifications etc. were made. The primary observations were made by mounting the material in water as well as in 1% aqueous cotton blue.

For staining the bacteria, gram-staining method was used. It is a very useful method for identifying and classifying bacteria into two major groups; gram- positive and gram- negative. In this process, the fixed bacterial smear is subjected to four different reagents in the order viz. crystal violet (primary stain), iodine solution (mordant), alcohol (decolorizing agent) and safranin (counter stain). The bacteria which retain the primary stain (appear dark blue or violet) are gram- positive, where as, those that loose the crystal violet (appear red) are gram- negative.

Components of Gram stain are as follows-

Crystal violet (Hucker's)–

Solution A	
Crystal violet	2.0 g.
Ethyl alcohol (95%)	20.0 ml.
Solution B	
Ammonium oxalate	0.8 g.
Distilled water	80.0 ml.

Gram's Iodine-

Iodine	1.0g.
Pottassium iodide	2.0g.
Distilled water	300.0ml.

Ethyl alcohol (95%)-

Ethyl alcohol (100%)	95.0ml
Distilled water	5.0ml

Safranin-

Safranin (2.5% solution in 95% ethyl alcohol)	10.0ml
Distilled water	5.0ml.

The biochemical tests for bacteria viz. Mannitol, Glucose, Lactose, Methyl Red, Voges Proskauer, urease, Citrate, etc. were taken and different characters were studied. The procedure used for biochemical tests are as follows-

1) Mannitol test- The mannitol broth is prepared. Tubes with mannitol broth are inoculated with different bacterial cultures each. Inoculated tubes are incubated at 35^0C for 24-48 hours. Change in color as well as appearance of bubbles (due to production of gas and acid) were recorded. Positive test is indicated by the color change from red to yellow and in negative test color remains red.

Components of Mannitol broth (pH 9.3)-

Mannitol	15.0g
Magnesium Sulphate	0.2g
Dipotassium Hydrogen Sulphate	0.5g
Calcium Sulphate	0.1g
Calcium Carbonate	5.0g
Sodium Chloride	0.2g
Distilled water	1000.0ml.

2) Glucose and Lactose test- In these tests, glucose broth and lactose broth containing test tubes are inoculated with bacterial cultures. Inoculated test tubes are incubated at 35^0C for 24-48 hours. Fermentation of glucose and lactose results in the production of acid or acid and gas. Negative reaction is indicated by the unchanged red color. In the positive reaction red color changes to yellow due to acid production.

Composition of fermentation medium are as follows-

Peptone	10.0g
Carbohydrate (Glucose/ Lactose)	5.0g
Sodium Chloride	15.0g
Phenol Red	0.018g
Distilled water	1000.0ml
pH	7.3

3) Methyl Red and Voges Proskauer tests- The Methyl Red (MR) and Voges Proskauer (V-P) tests are used to differentiate two major types of facultatively anaerobic enteric bacteria that produce large amounts of acid and those, that produce the neutral end product acetoin. Both these are performed simultaneously because; they are physiologically related and are performed on the same medium, MR-VP broth. MR-VP tubes are inoculated with bacterial cultures. One tube is kept as control. All the tubes are incubated at 35^0C for 48 hours. For MR test, 5 drops of Methyl Red indicator are added in each tube and color change is observed. No color change i.e. red indicates a positive test (because Methyl Red indicator in the pH range of 4 will remains red), while change from Methyl Red to yellow is a negative test.

For V-P test, 12 drops of V-P reagent I and 2-3 drops of V-P reagent II are added in the inoculated tubes. Tubes are shaken gently for 30 seconds and kept as it is for 15-30 minutes. The development of crimson to ruby pink (red) color, is a negative test.

Components used in the MR-VP broth (pH- 6.9) are as follows-

Peptone	7.0g
Dextrose	5.0g
Potassium Phosphate	5.0g
Distilled water	1000.0ml
Methyl Red indicator-	
Methyl Red	0.04g
Ethyl Alcohol (absolute)	40.0ml
Distilled water	60.0ml
V-P reagent I -	
KOH	40.0g
Distilled water	100.0ml
V-P reagent II -	
α- napthol	5.0g
Absolute alcohol	100.0ml

4) Urease test- Urease test is performed by growing the test organisms on urea broth or agar medium containing the pH indicator Phenol Red (pH 6.8). Urea agar slants are inoculated with bacterial organisms. Inoculated slants are incubated for 24-48 hours at 37^0C. The slants are examined, red color indicates presence of urease and yellow color means absence of it.

Preparation of Urease agar medium (pH 6.8) whose constituents are as follows-

Peptone	1.0g
Sodium Chloride	5.0g
Potasssium Monohydrogen Phosphate	2.0g
Agar-Agar	20.0g
Distilled water	1000.0ml

Phenol Red Indicator-

Phenol Red	125.0mg
Water	62.5ml

5) Citrate test- Citrate test is used to differentiate among enteric bacteria on the basis of their ability to utilize or ferment citrate as a sole carbon source. Simmon's citrate agar slants are inoculated with bacterial cultures. One tube is kept as un-inoculated comparitive control. Inoculated and un-inoculated slants are incubated at 37°c for 48 hours. Slants are observed for color change and growth. If growth is visible on the surface and the medium color is blue, the test is positive otherwise test is citrate negative.

Preparation of Simmon's Citrate Agar (pH 6.9)

Ammonium Dihydrogen Phosphate	1.0g
Dipotassium Phosphate	1.0g
Sodium Chloride	5.0g
Sodium Citrate	2.0g
Magnesium Sulphate	0.2g
Agar-Agar	15.0g
Bromothymol Blue	0.8g
Distilled water	1000.0 ml

Measurements - The micrometry of all materials were made with the help of Japan Erma type of ocular micrometer. The ocular scale was calibrated with the stage micrometer and was reduced to 1 division. Thus, each eyepiece of 5x, 10x and 15x magnification was calibrated with the objectives of 10x, 45x, and 100x (oil immersion) magnification. Accurate measurements of colony, mycelium, hyphae, hyphopodia, conidiophores, vesicle, septate conidia, phialides, metulae, spores and cells were taken which are important in identification of the spores as well as species.
Identification- Identification of fungi was done with the help of recent and up to date literature [Plate fig. nos. I to IX, Gravity petridish method]. Following literature was used for identification viz. Dematiaceous Hyphomycetes- Ellis (1976), More

Dematiaceous Hyphomycetes- Ellis (1976), Hyphomycetes- Subramanium (1967), Illustrated Genera of Imperfect Fungi- Barnet (1973), Mucorales of India- Tandon (1968). Identifying Filamentous Fungi - St. Germain, G. and R. Summerbell. (1996), Laboratory Handbook of Dermatophytes - Kane, J. et al. (1997), Medically Important Fungi a Guide to Identification- Larone, D.H. (1995), Medical mycology- Chester, W. Emmons, Chapman, H. Binford, John P.UTZ. K. J. Known-Chung (1977) etc. An Internet facility was also used for reference work. For the identification of bacteria, Bergey's manual of systematic bacteriology- James, T. Statey., Marvin, P. Bryant., Norbert, Norbert, Pfenning, John, G. Holt (1989), was used.

Tables- All the genera and species were tabulated as per the dates of observation. Their individual percentage was calculated. The dominant genera were recorded. To show comparison between them, various tables, histograms and pie-charts are prepared. This gives an idea of occurrence of various biopollutants and their concentration in air. The observations were prepared season-wise and month-wise, in different tabular forms. The bacteria are tabulated as per the dates of observation and in season-wise and month-wise manner. Different colony characters and biochemical tests are also tabulated. Various histograms and pie-charts are prepared.

Photomicrography- The important characters i.e. reproductive characters of the fungal materials were, photomicrographed by Nikon camera model E8-400 at Department of Botany, Shivaji University, Kolhapur. The photomicrography was done with the help of 5x, 10x and 15x magnifications of eyepiece in combination with 5x, 10x, 45x and 100x or oil immersion. The photoplates were made, named and numbered from plate fig. no. 1 to plate fig. Nos. 11 The exposed petriplates showing various colonies were also photomicrographed by Practica MTL- 50 type of camera having a Photomicrography unit with 6.4x 12.4 and 40x 12.4 magnifications. The photograph plates were made, named and numbered from plate fig.no.1 to plate fig.nos.15.

Citations and References: In citing the reference in journals, generally the list of the references or bibliography is given at the ends in which it includes title of article, date, volume or first and last pages. Journal is typed in the italic type, volume in bold type and the part numbers following the volume in the normal type. In the present investigation, the references which are cited in the work have been listed. The system has been followed here as Author's name was followed by fore name (initials), the year and then the title of the article following the journal abbreviations, the volume

number and the pagination. The journal abbreviations are underlined and volume number has been double underlined. All the volumes, pagination, title have been checked and confirmed. In citing the reference of book Author's name was followed by fore name, the year and then the title of the book, following the name of publisher and publication place.

Deposition of slides- The semi permanent micropreperations were cleaned properly, labeled with black India ink and arranged alphabetically in wooden cabinets and were deposited in Mycological Herbarium, Botany Department, Yashavantrao Chavan Institute of Science Satara, numbered from 1 to 52.