American Industrial Hygiene Association Journal

ISSN: 0002-8894 (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/aiha20

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To cite this article: KATHY L. MORRING , W.G. SORENSON & MICHAEL D. ATTFIELD (1983) Sampling for Airborne Fungi: A Statistical Comparison of Media, American Industrial Hygiene Association Journal, 44:9, 662-664, DOI: 10.1080/15298668391405526

To link to this article: https://doi.org/10.1080/15298668391405526

Am. Ind. Hyg. Assoc. J. 44(9):662-664 (1983)

Sampling for Airborne Fungi: A Statistical Comparison of Media

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Four broad spectrum media employed for enumeration of fungi from air were compared to determine which would yield the highest number of colony forming units when simultaneously sampling air from the same environment. The four media tested were: Inhibitory Mold Agar, Littman Oxgall Agar, Rose Bengal-Streptomycin Agar, and Sabouraud Dextrose Agar. The number of colony forming units produced on the media were compared by an incomplete four-factor factorial design (day, time, sampler and medium). Analysis of variance of the data indicated that highly significant variation was associated with collection medium and different sampling days. Comparisons among the media were performed by Duncan's multiple range test which demonstrated significantly lower values on Littman Oxgall as compared to the other media. There was no significant difference in the number of colonies produced on Rose Bengal-Streptomycin Agar, Inhibitory Mold Agar, and Sabouraud Dextrose Agar. For a number of reasons, Rose Bengal-Streptomycin Agar is our medium of choice for broad spectrum aeromycological sampling.

Introduction

A variety of fungal spores can be found in the indoor environment. Species of *Cladosporium, Alternaria, Mucor, Aspergillus,* and *Penicillium* are common and capable of reproducing indoors. Suitable niches for growth and sporulation include stored food, house plants, air conditioners, humidifiers, cold air vaporizers, books and papers, carpets, and damp areas. In addition, several work environments are conducive for the growth and sporulation of fungi, *e.g.,* farming, grain handling, mushroom cultivation, insect rearing, and pharmaceutical manufacturing. Fungal spores may be allergenic to some individuals, and more importantly, certain species cause invasive disease, particularly in immunosuppressed patients. The National Institute for Occupational Safety and Health (NIOSH) has the responsibility to assess all hazards to workers. The first step in the assessment for aerobiological content is to determine which organisms are present and to quantitate their densities. For this, agar media that will support the growth of the broadest variety of organisms and yield the highest number of colonies from the air sample are needed. Rogerson[1] advocated the use of rose bengal in media designed for aeromycological studies. Subsequent workers[2] have suggested that the use of rose bengal may be undesirable. Furthermore, studies comparing media for sampling airborne fungi[1-3] generally lack adequate statistical evaluation. Therefore, the objective of this study was to compare several media commonly used for enumeration of fungi by statistical analysis. NIOSH frequently receives requests to determine the airborne fungal concentration in indoor environments. Results from studies of this type will be helpful in developing methodology for these assessments.

Methods and Materials

Instrumentation

The Andersen 6-stage Viable Particle Sampler (Andersen Samplers Incorporated, Atlanta, GA) is a multi-orifice cascade impactor which is normally used to measure the concentration and particle size distribution of aerobic bacteria and fungi in the ambient air. Air enters the sampler at the inlet, cascades through a series of orifice stages with successively higher orifice velocities from stages 1 through 6. It is calibrated so that all particles of a particular aerodynamic size range will collect on the designated stage (an agar plate) so that the results represent the lung penetration capacity of that fraction.

Sampling Methods

Four Andersen 6-stage Viable Particle Samplers were calibrated to 28.3 liters per minute (1.0 cfm) and placed on a cart. Their positions on the cart and the cart's position relative to the sampling sites were constant throughout the study. The sampling sites were the lobby of the Mayo Memorial Hospital (University of Minnesota, Minneapolis, MN) and the receiving area near the loading dock of the NIOSH building in Morgantown, West Virginia. The samplers were dismantled, swabbed with 70% 2-propanol, and loaded with the media designated for each trial. The disinfection procedure was repeated between trial runs. The vacuum pumps for the samplers were connected to an electrical strip with a single on off switch controlling simultaneous starting and stopping of the motors. This, in combination with a stopwatch, ensured equivalent sampling duration for each sampler and trial. Two types of controls were used during the study. First, plates from each batch were preincubated to ensure sterility and second, plates were loaded in the sampler without operation of the pump to determine whether the sampler itself contributed to colony counts.

The petri plates were incubated at 31°C. Colonies were counted daily until the plates were 6 days old. Results were expressed in colony forming units per cubic meter of air (CFU/m^3). Often the number of colonies on any given plate decreased with time because of overgrowth by the faster growing organisms. For this reason, the highest count for each plate was used.

The mention of company names or products does not constitute endorsement by the National Institute for Occupational Safety and Health.

TABLE I
Comparison of Media for Sampling for Fungi (Minneapolis Site)[A]

Media	Mean[B] CFU/m³	No. Samples
RBS	340.2 ± 271.7	13
IMA	308.5 ± 304.4	14
LO	91.4 ± 36.9[C]	14

[A]Sample volume = 10 m³.
[B]Mean ± standard deviation.
[C]Colony counts were significantly lower than on RBS or IMA (Duncan's multiple range test).

TABLE II
Comparison of Media for Sampling for Fungi (Morgantown Site)[A]

Media	Mean[B] CFU/m³
SDA	128.1 ± 164.5
RBS	123.3 ± 157.6
IMA	116.9 ± 142.8
LO	39.1 ± 40.7[C]

[A]14 samples; sample volume ≈ 20 m³.
[B]Mean ± standard deviation.
[C]Colony counts were significantly lower than on RBS, IMA, or SDA (Duncan's multiple range test).

Preliminary work in the indoor environment indicated that a sampling period of 10-20 min at 28.3 Lpm would yield countable plates and allow computation of the number of trial repetitions necessary to obtain a critical value of $\alpha = 0.05$. An estimate of population variance, derived from previous experience indicated that for comparing four media, approximately 14 replicates were needed. Sampling was performed at the same times each day for 7 days. Two sampling trials were run each day at 11:30 A.M. and 12:10 P.M. This divided the samples into two subgroups for each site: samples taken for the first trial of the day and those taken for the second.

Statistical Analysis

To verify the existence of an effect caused by the medium on the mean number of colony forming units, an incomplete four-factor factorial design was used. The four factors were day, time, sampler and medium. To explore the statistical significance of these factors, an analysis of variance was undertaken on the logarithms of the CFU/m³ (Statistical Analysis System, Parklawn Computer Center, Rockville, MD). Logarithms were used because of the large range in the observations and because it was felt that the factors probably acted multiplicatively rather than additively. To determine whether the differences detected among CFU/m³ could be attributed to the media, a Duncan's multiple range test among the media was performed.

Media

The following media were employed: Sabouraud Dextrose Agar (SDA),[4] Littman Oxgall Agar (LO),[5] Inhibitory Mold Agar (IMA),[6] and Rose Bengal-Streptomycin Agar (RBS).[1] The Sabouraud Dextrose Agar used at the Morgantown site contained penicillin (50 units/mL) and streptomycin (50 μg/mL). No antibiotics were used in SDA at the Minneapolis site.

Results

A summary of the results from repeated runs of the experiment at the Minneapolis site is presented in Table I. Since antibiotics were not included in the SDA plates used at the Minneapolis site and no attempt was made to differentiate between yeast and bacterial colonies in the Minneapolis samples, the results from SDA plates were omitted from comparison at the Minneapolis site. The study was repeated at the Morgantown site to permit comparison of SDA with the other media because SDA is a popular medium for fungal propagation. Results from the Morgantown site are presented in Table II.

The data in Table I indicate that much lower colony counts were obtained on LO than on IMA and RBS. Variations due to time of sampling and to sampler could easily have arisen through random causes (Table III). However, significant differences were found both for days and media. When sampling in the same environment, RBS and IMA yielded equivalent counts, each of which were significantly greater than that of LO (Tables I and III).

The data from the Morgantown experiment (Table II) were analyzed similarly. The effects of days and media were significant, while samplers and time of sampling again were not. LO yielded counts that were significantly lower (approximately 1/3) than those obtained on all other media. Differences between the number of CFU on RBS, IMA, and SDA were not observed ($p < 0.01$). Since the Minneapolis experiment had suggested that RBS and IMA were equivalent, a standard t-test was undertaken for those two media in the second experiment. This is permissible because it was a planned comparison based on evidence external to the second experiment. This more powerful test indicated that RBS and IMA yielded equivalent results.

TABLE III
Analysis of Variance of Air Sampling Data (Minneapolis Site)

Source of Variation	Sum of Squares	DF	F-Value
Residual	2.55	28	
Day	12.34	6	22.54[A]
Time	0.36	1	3.90
Sampler	0.07	3	0.25
Medium	8.37	2	45.89[A]

[A]Significant ($p < 0.01$).

Discussion

Analysis of variance of the data from the Minneapolis site identified sampling day and collection medium as significant sources of variation. It is not surprising that normal fluctuation in weather conditions and traffic in and out of the building could produce variable results. On the other hand, there was negligible variability among the samplers used. Duncan's multiple range test was used to make comparisons among the media. The results indicate that significantly fewer colonies developed on LO than on the other media (Tables I and II).

In 1950, Swaebly *et al.* compared 18 fungal media to determine which medium would yield the highest mold count when exposed to a particular environment by the sedimentation plate method.[3] Smith-Humfeld Salt Agar yielded the highest mold count, but this medium could not support the growth of such common Zygomycetes as *Rhizopus* and *Mucor*. Mehrlich's medium could support these fungi but resulted in lower colony counts than Smith-Humfeld Salt Agar. Therefore, neither medium was entirely satisfactory. Rogerson performed a similar comparison of eight fungal media using the Pady-Rittis slit sampler to determine which would yield the highest number of viable fungal counts from air samples as well as support the widest range of fungal types.[1] Rose Bengal-Streptomycin and Rose Bengal-Aureomycin were the best for counting and identification in that colonies remained discrete and sporulation usually occurred within 4-5 days. The inclusion of rose bengal in agar media restricted radial growth of hyphae and thus, minimized the confounding effect of spreading and overgrowth. Colonies on the other media were often difficult to count and sporulation was frequently poor. Thus, Rose Bengal-Streptomycin Agar met the study criteria of Rogerson because it supported high colony counts and yielded discrete, identifiable colonies. When representatives of the species collected from the air samples were inoculated onto this medium, no growth inhibition was noted. For these reasons, Rogerson selected Rose Bengal-Streptomycin as the medium of choice for subsequent aeromycological studies. In 1977, Burge *et al.* reported a study in which eight fungal media were compared using paired Andersen viable samplers.[2] Data were reported as the percent of plates positive for a given organism and thus, no colony count data were provided. Therefore, it is impossible to evaluate their data in terms of concentration per unit volume of air. When rose bengal was added to Potato Dextrose Agar in varying concentrations (1.5, 15, 150 mg/L agar), satisfactory results were not obtained. To conclude from this study that rose bengal is an undesirable ingredient in fungal media is misleading because rose bengal was not tested at a consistent concentration nor at the concentration most often used (50 mg/L). It is interesting that Burge *et al.*[2] also state that they have successfully used rose bengal in Potato Dextrose Agar in their laboratories for several years.

Our data suggest that RBS, IMA and SDA are essentially equivalent in the number of colonies developing on the plates. Littman Oxgall did not perform well in our study because the colonies were very small and colony counts were much lower than on the other media (Tables I and II). According to Littman,[5] the LO plates would produce higher colony counts than SDA if allowed to incubate for 6 weeks. It is impractical to hold plates for 6 weeks because most routine work and colony identification may be impaired because the hyphae are severely restricted. Therefore, LO is not recommended as a satisfactory broad spectrum medium for use in air sampling.

Even though the number of colonies produced on RBS, IMA and SDA were not statistically different, other factors influence the choice of media for sampling. Although IMA is simple to make and colony identification is not obscured by the translucent medium, it does not restrict colony spreading, and rapidly growing organisms (*e.g., Mucor*) can quickly render a plate uncountable. In our experience, SDA is similar to IMA both quantitatively and qualitatively (growth, spreading and sporulation).

The restricted radial growth of most fungal organisms on RBS is a great advantage, but this medium is not commercially available at the present time. In addition, rose bengal is sensitive to direct sunlight and must be shielded when sampling out of doors. Despite these disadvantages, RBS is an excellent fungal medium that yields high colony counts and impedes colony spreading. It is our experience that it is nearly as convenient to prepare as the other evaluated media. The use of RBS is not widely accepted and, in fact, has been discredited for aeromycological studies.[2] Our results concur with those of Rogerson.[1] Therefore, we believe that RBS is the medium of choice for broad spectrum aeromycological sampling.

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24 January 1983; Revised 13 May 1983