

Usefulness of using three different culture media for mold recovery in exposure assessment studies

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Received 20 July 2001; accepted 16 September 2002

Abstract

The choice of culture media when performing exposure assessment for mold quantification is difficult since no standard methods exist and, in many cases, there is no way to predict, before hand, the mold population present and thus, select the most appropriate media. The aim of this project was to compare the efficiency and usefulness of 3 widely used culture media during the recovery of molds in sawmills. Andersen microbial samplers (AMS) and all-glass impingers-30 (AGI-30) were used to sample 51 work sites within 17 sawmills. Rose Bengal Agar (RBA), Czapek Solution Agar (CZA) and Sabouraud Dextrose Agar (SDA) were used directly in AMS and to plate AGI-liquid. The results show that there was no significant difference between the three culture media concerning the counts and the species recovery. However, SDA often lead to non usable counts. The variations obtained with the different culture media when AGI-30 were plated in triplicate was also not significantly different. However, only 19% of the species recovered at one site were present on all culture media. We believe the use of various media increases the chances of obtaining valid counts and an adequate species recovery.

Introduction

During the past years, the role of airborne contaminants in various respiratory illnesses has become clearer. Exposure assessment of airborne molds is regularly performed in many working and domestic environments. Depending on the mold population of a highly contaminated environment (e.g. dairy barns, swine buildings, sawmills, peat moss processing) the approach for culture, enumeration and further identification of the molds may vary. There is no standard method for air analysis and, based on previous reports, there is a lack of consistency in the media used even if governmental associations such as the American Conference of Governmental Industrial Hygienists (ACGIH) recommends culture conditions for molds analysis (Burge et al., 1987). When an exposure assessment study is planned, the choice of the appropriate culture media for mold enumeration

and identification is often a challenge. The use of a wide variety of culture conditions should allow a better description of the mycoflora but adds to the complexity of the experimental protocol and multiplies the time required for the analyses of the samples.

Several studies have compared the use of various culture media in the evaluation of mycoflora in highly contaminated environments (Duchaine et al., 1999; Smid et al., 1989). In those cases, the colony count obtained is the information compared (Lacey and Dutkiewicz, 1976). Other studies describe the use of different combinations of culture media, but little or no comparison was performed (Comtois and Marcoux, 1999; Clark et al., 1983; Eduard et al., 1990). In other published exposure assessment studies, only one culture media was used such as Rose Bengal Agar (Berstein et al., 1983), Inhibitory mold Agar (Overberger et al., 1995), Malt extract Agar (Nielsen and Breum, 1995).

The aim of this paper is to compare the usefulness of the three culture media used in a study of exposure assessment to bioaerosols in eastern Canadian sawmills, whose data were previously published (Duchaine and Mériaux, 2000; Duchaine et al., 2000). Unlike the published papers, this one focuses on the methodology of air sampling and mold exposure assessment.

The main objective of the present study was to compare the usefulness of the utilization of 3 different culture media for *Penicillium* and other mold taxa recovery, quantification and identification from sawmills: Rose Bengal Agar with chloramphenicol (RBA), Czapek Solution Agar (CZA) with chloramphenicol and Sabouraud Dextrose Agar with chloramphenicol (SDA). Those media were chosen because of their importance in medical mycology and the abundant information provided in textbooks as regards to fungal colony morphology on those media (SDA and CZA) and for their ability to control colony size, making it possible to sample highly contaminated environments (RBA).

The secondary objectives are to 1) determine the efficiency of each of those three media and describe their strengths and weaknesses (the efficiency analyses include species recovery, count comparisons, rates of obtaining non usable counts) and to 2) evaluate the variability induced by the use of each medium.

Material and methods

Sawmills selection

The air contamination of the different working sites from 17 sawmills from various geographical regions from eastern Canada was estimated. In most cases, the working sites included debarking, sawing, sorting and planning. A total of 51 sites were sampled. Samplings were performed between the first of May and the thirty first of October of 1996 and 1997.

Environmental sampling

Culture media

Sabouraud Dextrose Agar (SDA) (Difco), Rose Bengal Agar (RBA) (Difco), and Czapek Solution Agar (CZA) (Difco) supplemented with chloramphenicol (50 mg/l) were used for molds culture. Yeasts were also counted, when present. All media were autoclaved at 121 °C for 12 minutes.

Air sampling

Ambiant air sampling was performed. Samplers were placed on a Table, 1 m above the floor, as near as possible to a working area. When possible, all doors were kept closed and the sampling apparatus were kept away from ventilation sources. Two types of air samplers were used at each sampling site: 3 six-stage Andersen microbial samplers (Andersen 1958) (AMS) (Graseby Andersen, Atlanta, GA, USA) and 3 all glass liquid impingers-30 (May and Harper, 1957) (AGI-30) (Ace Glass Inc., Vineland, NJ, USA). The AMS were used at a sampling flow of 28.3 l/min. They were loaded with SDA, RBA and CZA for a 30 seconds sampling. The 3 AGIs contained 20 ml of sterile saline solution (0.85% NaCl) and used at 12.5 l/min for 16 minutes.

The samples from AGIs were measured (to evaluate evaporation) and recovered. The impingers were then washed with a volume of sterile saline solution containing 0.1% Tween 80 to complete the sample volume to 30 ml. The final sample contained about 0.03% Tween 80. One milliliter of this 30-ml volume was diluted in 9 ml of sterile saline solution (10^{-1}) and this was repeated to a 10^{-3} dilution. Each sampling suspension (3/site) was diluted in triplicate. One hundred microliters of the dilutions (10^0 to 10^{-3}) as well as 0.5 and 1 ml of the non-diluted AGI liquids were plated on different media. The SDA, RBA and CZA plates were incubated at 30 °C for 7 to 10 days. The culture conditions for the plates used in the AMS were the same. Depending on the mold concentration or the species present, the sampling device that allowed a better analysis of the sample was retained (AGI or the AMS). For instance, if the colonies were small or in small numbers on each AMS plate, this device could be used. When the colony sized led to growth confluence or when the number of colonies was too high, AGI were used at the appropriated dilution rate. Most of the time, only one sampling device led to a usable colony count. To allow media efficiency comparison, the values were taken from the same type of sampling device for a given site: each site was taken independently from the others. Since we are aware of the fact that different sampling devices are not comparable, no comparison was performed between values from different sites (that could come from different sampling device). The comparisons were performed within a same site.

Molds isolation, quantification and identification

Once colonies were counted, all the different ones were isolated if growth was not too confluent. Every macroscopically different mold colony was isolated, subcultured and kept at 4 °C for later identification. Since sampling was performed over several months, different colonies were isolated each time. We could end up with similar colonies, isolated from different samples. For *Penicillium* species, John I. Pitt's guide for *Penicillium*, *Talaromyces* and *Eupenicillium* genera identification was used (Pitt, 1979). This identification and taxonomic key is based on the microscopic and macroscopic observation of strains on 3 different culture media, at 3 different temperatures: Czapek Yeast Autolysate Agar (CYA), at 5, 25 and 37 °C and Malt Extract Agar (MEA) and 25% glycerol nitrate Agar (G25N), both at 25 °C. For *Aspergillus* species and teleomorphs, the «Laboratory guide to common *Aspergillus* species and their teleomorphs» was used (Klich and Pitt, 1994). This identification system is based on 3 culture media: Czapek yeast extract Agar with 20% sucrose (CY20S) at 25 °C, CYA at 25 and 37 °C, and MEA at 25 °C. The other taxa were identified using reference books (Samson et al., 1996; Wang and Zabel, 1990; St-Germain and Summerbell, 1996; Larone, 1995).

Statistical analysis

Since normality assumptions were not met, comparisons were performed by randomized block analysis on data transformed by rank. For the variation comparisons between AGI-30 samples and analysis replicates, paired t-tests were performed on variation coefficients since normality was met within sample and analysis replicates. The data were analyzed using the statistical package program SAS (SAS Institute Inc., Cary, NC). All reported p-values are two-sided. The results were considered significant if p-values were <0.05.

Results

The total counts obtained for each site (CFU/m³) from each culture medium are shown in Table 1. Each number represents the mean of 9 plates for AGI sample (3 AGIs plated in triplicates) or a single value for Andersen samples (sum of the 6 stages). Nine sites had more appropriate values (appropriate number of colonies per plate) with Andersen than with the

AGI sampling. All other values were taken from AGI samples plates.

Table 2 shows different comparisons performed on the results obtained from the different culture media. The first value gives the number of sites (out of 51) where the colony count was not possible (confluent growth). The number of sites where a certain medium value was the highest obtained or the only one available to also given. The easiness with which small morphology differences between species and genera are observable may very depending on the characteristics of the medium: the % of colonies counted that led to an isolation and identification is therefore given, for each medium (mean $\pm$ SD). A total number of species was identified for each site. The ratio of the total species found in one site that were isolated on each medium is also given in Table 2 (mean $\pm$ SD). The mean % of species found in one site that were common to all media is given.

We verified the variability of the AGI samples that were taken in triplicate at the same time and that were used for the total count for 42 of 51 sites. Twelve percent of these sites showed a significant variability between samples (AGI), 24% showed a significant difference between the medium used for the count obtained ($p = 0.0001$). Among those sites that show a difference, SDA gave the highest count in 3 cases, RBA in 2 cases and CZA in one case (data not shown). We verified the variation coefficient of the 3 AGI samples (taken at the same time) and for the analysis replicates (same AGI liquid diluted and plated in triplicate, for each medium). The variation coefficients (CV) for the AGI samples were not statistically different between the media (mean CV $\pm$ SD): $51 \pm 36\%$, $56 \pm 47\%$ and $47 \pm 22\%$ for RBA, CZA and SDA respectively, even though there was a tendency for CZA-plated samples to be more variable than the SDA-plated ones ($p = 0.06$). Those coefficients were also not statistically different for the analysis replicates: $44 \pm 39\%$, $47 \pm 40\%$ and $40 \pm 31\%$ for RBA, CZA and SDA, respectively. The lower variation coefficients of replicates from a same sample than from different samples was not surprising because the differences observed between samples are more likely to be greater than between replicates in the analysis of the same sample.

One thousand seven hundred strains were isolated, grouped according to colony morphology and identified from the sawmill sites, giving a total of 63 different genera and species. Table 3 shows the taxa identified from the sawmills and the total number of

Table 1. Total counts (mean CFU/M³) for each of the 51 sawmills sites as counted on Rose Bengal Agar (RBA), Czapek Solution Agar (CZA) and Sabouraud Dextrose Agar (SDA).

Sawmill	Debarking			Sawing			Sorting			Planing		
	RBA	CZA	SDA	RBA	CZA	SDA	RBA	CZA	SDA	RBA	CZA	SDA
1	36 000	15 333	22 000	–	–	–	24 000	14 000	110 000	–	–	–
2	2 500	2 000	4 167	1 000	2 167	6 667	667	915	2 000	1 667	1 167	1 833
3	–	–	–	33 833	Not valid	34 667	2 233	2 500	5 200	7 667	500	400
4	–	–	–	483	417	983	756	387	885	–	–	–
5	3 783	3 800	4 667	533	733	2 133	30 000	24 000	Not valid	1 500	1 800	Not valid
6	186 667	213 333	190 000	20 667	23 333	42 333	8 133	8 333	7 616	Not valid	Not valid	30 000
7	–	–	–	3 167	Not valid	3 000	–	–	–	915 [A]	Not valid	Not valid
8	2 958 [A]	Not valid	2 925 [A]	1 831 [A]	845 [A]	Not valid	5 704 [A]	845 [A]	Not valid	423 [A]	141 [A]	Not valid
9	36 427	20 750	38 167	5 050	1 150	8 767	–	–	–	2 254 [A]	2 183 [A]	Not valid
10	22 750	22 500	39 250	3 732 [A]	1 972 [A]	Not valid	1 549 [A]	1 127 [A]	Not valid	5 050	4 500	2 500
11	7 833	6 000	6 333	2 367	467	2 267	–	–	–	1 833	2 600	2 208
12	1 433 333	1 750 000	1 266 667	1 008 333	1 266 667	1 833 333	–	–	–	–	–	–
13	–	–	–	3 300	4 000	2 450	1 467	1 767	3 233	–	–	–
14	1 967	2 033	2 400	1 133	1 633	2 100	–	–	–	800	1 300	1 200
15	–	–	–	90 000	87 500	80 000	–	–	–	1 950	Not valid	Not valid
16	1 600	1 125	1 250	2 617	1 667	4 933	–	–	–	2 465	2 887	Not valid
17	5 070 [A]	4 437 [A]	Not valid	Not valid	Not valid	Not valid	–	–	–	563	Not valid	Not valid

The site where no precise count could be obtained (confluent growth leading to inexact colony count) are shown as «not valid». When a specific working site was not present or not sampled in a sawmill it is shown by (–).
[A] = from Andersen sampler.

Table 2. Comparison of the counts and species identification efficiency for Rose Bengal Agar, (RBA), Czapek Solution Agar (CZA) and Sabouraud Dextrose Agar (SDA) for 51 sawmills sites.

	RBA	CZA	SDA
Sites where the value was not valid (no count was obtained) n (%)	2 (4)	8 (16)	14 (28)
Sites where the value obtained was the highest or the only one n (%)	20 (40)	7 (14)	20 (40)
% of colonies counted that led to an isolation and identification Mean (SD)	63.3 (28.7)	69.5 (32.9)	71.9 (18.8)
% of the total species found at one site that was isolated and identified on each media Mean (SD)	57.5 (20.5)	51.7 (16.4)	58.2 (24.2)
% species that were found in one site on all the 3 media Mean (SD)		19.2 (14.8)	

sites where a given taxa was isolated and identified from each media. If a certain taxon was identified on one of the 9 plates available (or one of the 6 Andersen plates), a site was then counted as positive for this species.

Discussion

The 3 culture media used gave similar results: no significant difference was observed in term of the percentage of colonies counted that led to an identification, the number of total species found at one site that were isolated and identified on each medium. Only 2 sites out of 51 had an invalid measure with RBA. This can be explained by the inhibitory effect of rose Bengal which limits colonies size and prevents confluence. The invalid results were much higher when SDA plates were analyzed.

CZA cultures were difficult to analyze since the isolation of colonies was based on morphology. On this medium, the mycelium and the sporulation are very sparse. Exudate and diffusible pigments are also very rare. Many colonies from CZA had to be isolated on a different medium before any grouping or identification could be performed.

SDA frequently gave the only or the highest count (40% of the sites). However, it more often gave an invalid result (28% of the sites). We also observed that

SDA led to invalid counts at 8 sites out of 9 that were taken from Andersen sampler. We think that since SDA leads to larger colony sizes, the rapid confluence of the growth when the dish contained fewer colonies made us read the counts at higher dilution rates: the correction factor for the higher dilutions increases the overestimation of the counts since, when the colonies are big, we can only count a few colonies/plate which is not suitable for statistical analyses. The higher counts obtained with SDA could be an artefact of more frequent overestimation. The incubation time could not be reduced since the colony characters are very hard to distinguish before 7 days of growth. Therefore, the frequent confluence of the growth made SDA a less valid choice in highly contaminated environments and a useless media to be used with AMS devices: the usefulness of this medium is debatable for aerobiological analyses.

RBA gave good overall results with 4% of invalid counts, 40% of the counts being the highest or only one and a good rate of species recovery.

It is very difficult to determine if some species were more easily recovered from one medium than one other. The values obtained (Table 3) showed a good distribution of the species on each medium. However, for taxa other than *Penicillium* and *Aspergillus*, CZA seems to be less efficient (2 taxa only were recovered more often from this medium). Some species such as *P. myczinskii* and *P. spinulosum* were isolated more often from RBA than the other medium. This could be explained by the lower sites with invalid counts for this medium. In average, only 19% of the species found at one site were isolated from all 3 media. As shown in Table 3, each medium allowed the recovery of taxa that were not isolated from the other media, this renders very difficult the elimination of any of the 3 media from a standard protocol for optimal biodiversity assessment.

Conclusion

The study showed the different weaknesses and strengths of three commonly used culture media for exposure assessment in highly contaminated environments. We believe the use of various culture media increases the chances of obtaining valid counts and an adequate species recovery.

Table 3. Number of sites where each taxa was isolated and identified at least on one replicate (possibility of 51 sites).

Taxa	RBA	CZA	SDA	Taxa	RBA	CZA	SDA	Taxa	RBA	CZA	SDA
<i>Pen. aculeatum</i>	1	0	0	<i>Pen. purpurescens</i>	4	9	10	<i>Alternaria</i> sp.	0	1	3
<i>Pen. aurantiogriseum</i>	3	6	7	<i>Pen. purpurogenum</i>	1	0	5	<i>Arthrinium</i> sp.	0	1	4
<i>Pen. canescens</i>	7	10	9	<i>Pen. raistrickii</i>	15	14	19	<i>Aspergillus</i> sp.	0	0	2
<i>Pen. chrysogenum</i>	0	3	1	<i>Pen. roquefortii</i>	2	2	2	<i>Bauveria</i> sp.	1	0	2
<i>Pen. citreonigrum</i>	0	2	0	<i>Pen. roseopurpureum</i>	4	2	2	<i>Chrysonilia</i>	1	0	0
<i>Pen. crustosum</i>	9	5	8	<i>Pen. rugulosum</i>	18	17	21	<i>Clado. carionii</i>	0	1	0
<i>Pen. echinulatum</i>	1	1	1	<i>Pen. simplicissimum</i>	1	1	1	<i>Cladosporium</i> sp.	4	5	4
<i>Pen. fellutanum</i>	20	15	18	<i>Pen. spinulosum</i>	33	25	28	<i>Eurotium</i> sp.	2	1	2
<i>Pen. glabrum</i>	11	3	3	<i>Pen. thomii</i>	0	3	0	<i>Exophiala</i> sp.	5	6	4
<i>Pen. islandicum</i>	1	1	1	<i>Pen. variable</i>	1	2	1	<i>Fusarium</i> sp.	1	1	0
<i>Pen. janczewskii</i>	0	0	1	<i>Pen. velutinum</i>	4	4	1	<i>Geotrichum</i> sp.	0	0	1
<i>Pen. janthinellum</i>	0	0	1	<i>Pen. verrucosum</i>	2	0	0	<i>Lecythophora</i> sp.	1	0	0
<i>Pen. jensenii</i>	3	3	1	<i>Pen. viridicatum</i>	2	4	0	<i>Paecilomyces</i> sp.	4	5	7
<i>Pen. lignorum</i>	2	0	0	<i>Pen. waksmanii</i>	4	10	8	<i>Phialophora</i> sp.	1	0	0
<i>Pen. lividum</i>	2	1	5	<i>Eupenicillium</i> sp.	18	17	10	<i>Phoma</i> sp.	1	0	0
<i>Pen. melinii</i>	4	3	3	<i>A. flavus</i>	1	0	0	<i>Rhizopus</i> sp.	3	1	0
<i>Pen. minioluteum</i>	1	0	0	<i>A. fumigatus</i>	8	11	8	<i>Sporothrix</i> sp.	1	1	2
<i>Pen. montanense</i>	2	1	0	<i>A. niger</i>	3	3	6	<i>Trichoderma</i> sp.	21	15	14
<i>Pen. myczinskii</i>	32	23	22	<i>A. terreus</i>	0	0	1	<i>Verticillium</i> sp.	1	0	0
<i>Pen. novae-zeelandiae</i>	1	0	0	<i>A. versicolor</i>	1	4	1				
<i>Pen. oxalicum</i>	0	1	0	<i>Acr. butyri</i>	1	0	0				
<i>Pen. piceum</i>	0	1	0	<i>Acremonium</i> sp.	0	0	1				

Acknowledgements

The authors are thankful to Mr. Serge Simard for statistical analysis. This work was supported by Quebec Institute of Research on Occupational Health and Safety (IRSST). Caroline Duchaine was supported by an IRSST postdoctoral fellowship.

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