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RECOVERY OF FUNGI FROM SEEDED SPUTUM SAMPLES: EFFECT OF CULTURE MEDIA AND DIGESTION PROCEDURES

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SUMMARY

The effect of digestion procedures and of various culture media on the isolation of respiratory mycotic agents from seeded sputum samples was investigated. The digestants (N-acetyl-l-cysteine and pancreatin), did not affect viability of the microorganisms studied. Among the various culture media used, 4 appeared to constitute a suitable battery, as follows: Sabhi, modified Sabouraud, yeast extract agar and Littman plus bird seeds. In spite of high contamination rate, Sabhi was effective for all fungi tested, except **P. brasiliensis.** Modified Sabouraud and yeast extract agars produced adequate growth of **H. capsulatum, P. brasiliensis, N. asteroides, A. fumigatus** and **S. schenckii.** The yeast extract agar exhibited less contamination than modified Sabouraud. Littman bird-seeds agar allowed prompt recognition of pigmented **C. neoformans** colonies. No improvement in recovery rates were noticed with other culture media.

INTRODUCTION

The primary isolation of fungi causing pulmonary diseases is not always successful [7,9]. The clinical sample most often examined is sputum, which normally has a certain concentration of bacteria and yeasts [2]. Growth rate of these contaminating organisms exceeds that of most pathogenic fungi which require over 10 days to produce detectable colonies [14]. Although the addition of antibacterials and mold inhibitors has resulted in improved recovery rates [9], isolation of the causative agent still represent a problem [5,6]. Culturing of repeated samples in a battery of selective and non-selective media has been recommended to achieve higher isolation rates [9,16]. Digestion and concentration of the mucous samples also appear to improve the situation [5,10,13]. The variety of media and treatments recommended, hinders easy selection of the most appropriate combination of procedures.

This study was undertaken to determine the effect of 2 digestion procedures and of a battery of culture media in the isolation, from seeded sputum samples, of those fungi known to cause respiratory disease in South America.

MATERIALS AND METHODS

Strains

Two isolates each of **Aspergillus fumigatus, Cryptococcus neoformans, Histoplasma capsulatum, Nocardia asteroides, Paracoccidioides brasiliensis** and **Sporothrix schenckii** were studied. All the isolates had been recovered from human sources in this laboratory, as shown in Table I. Strains were maintained on Sabouraud's dextrose agar (BBL Cockeysville, MD) at room temperature with monthtly subcultures. At time of the study, the dimorphic fungi were transferred to Kelly's hemoglobin agar slants [8], with incubation at 36C to obtain the tissue (yeast) form. The monomorphic fungi were transferred to fresh Sabouraud's agar slants and incubated at room temperature (22 ± 3 C).

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Young 5-days old cultures were used for the experiments.

The entire growth on the slant was suspended in 9.9 ml of sterile buffered saline pH 6.8, homogenized by mechanical agitation in a Vortex mixer and adjusted to a turbidity equal to that of McFarland +1 tube. In the case of **N. asteroides,** previous homogenization by grinding with morter and pestle was required. Preliminary trails had indicated that in the case of **P. brasiliensis** and **H. capsulatum** the above turbidity gave adequate colony counts (50-100 colonies/0.1 ml). With the remaining fungi, a further 1:100 dilution was required. Fungal suspensions were used within 1 hour of preparation and homogenized by mechanical agitation before incorporation into the samples. Viability of the inoculum was determined by transferring 0.05 ml of the suspension to each of 2 Sabouraud's dextrose agar slants, with incubation at room temperature for 2 weeks. One ml of the suspension was used to seed 15 ml of pooled sputum.

Sputum Samples

Samples (*) which had been found negative for acid-fast bacilli were collected daily, pooled, homogenized in a blender for 5 minutes and adjusted to a quantity of 300 ml with buffered saline, pH 6.8 [16]. The homogenized specimen was then transferred to individual screw-capped centrifuge tubes in 15.0 ml amounts, and frozen. The pooled sample was used within 10 days and a single lot employed for each experiment.

Culture Media

All fungi, except **C. neoformans,** were plated in the following media: **Sabhi agar** (Sab) with 100 mg/liter chloromycetin, (Difco Laboratories, Detroit, MI) [7]. **Modified Sabouraud dextrose agar** (M) (Mycosel BBL, Cockeysville, MD), with cycloheximide (0.4 g/liter) and chloromycetin (0.5 g/liter). **Modified Sabouraud** (M5F), plus **250 μg/ml of 5 fluorocytosine** (Hoffman-La Roche, Nutley, N.J.) [5]. **Yeast extract agar** (Y) with cycloheximide (0.5 g/liter), penicillin (40 U/ml) and streptomycin (20 mg/liter, (14). **NH_4OH- yeast extract agar** (MY) with 50 mg/liter of chloromycetin (15). **Sabouraud's dextrose agar** (S) plus 50 mg/liter chloromycetin (4). For **C. neoformans,** media used were Sab, MY, S and, in addition **Littman bird-seeds agar** (LBS) with 30 mg/liter streptomycin [1]. All media were distributed in Petri dishes, kept in the refrigerator and used within 1 week of preparation.

Digestion — Decontamination Procedure

For each one of the 6 fungi, 1.0 ml of the standarized spore suspension was used to separately inoculate 3 different tubes with the pooled sputum sample. After thorough mixing in a Vortex, the mixtures were treated as recommended by REEP & KAPLAN [10,11]. Tube 1, served as the inoculum's control; tube 2 was treated with pancreatin [14] and tube 3 was treated with n-acetyl-l-cysteine (NAC) in 2.94% sodium citrate [12]. A fourth tube which received no fungus and remained untreated, served as control of the sample lot. After completion of the digestion period, the samples were centrifuged in the cold at 1.900 x g for 30 minutes. The supernate was discarded and the sediment used for the inoculation of the various culture media. Aliquots of 0.01 ml were delivered by a calibrated loop; the inoculum was then spread throughout the surface of the plate.

All cultures were kept at room temperature (22 ± 3 C), in the dark for 3-4 weeks, with weekly readings. Colonies were counted and degree of contamination recorded. In the case of **C. neoformans,** formation of pigmented colonies in the appropriate medium was also noticed.

Statistical Analyses

Significance of the data was determined by Students T test.

RESULTS

Twenty-four different pooled samples were prepared and a corresponding number of experiments performed. Half of them were carried out with one set of isolates and half with the remaining. There were no differences according to the isolates; consequently, the data were pooled and treated as one. Seven samples had to be discarded due to massive bacterial growth on both control and seeded plates. None of the remaining 17 pooled samples proved positive by culture for the agents employed in the study.

(*) Specimens were kindly provided by the Public Health Laboratory, Medellín, Colombia

The effects of digestion procedure and of culture media on the recovery of the various fungi are shown in Tables II and III. Statistical analyses of the figures above revealed no significant differences in recovery rates according to digestion procedure. Irrespective of the fungus and although with the same medium mean colony counts varied somewhat, the differences were not significant. On the other hand, type of culture medium influenced recovery and some media produced more abundant growth than others. Average colony counts for the 3 digestion procedures were obtained for each of the culture media used (Table IV). Significant differences ($P \leqslant 0.05$) were recorded as follows: **H. capsulatum** — SAB was superior to the other 4 media; M, Y and MY were better than M5F; **P. brasiliensis** — No growth was obtained in M5F; M, Y and MY proved superior to SAB; Y was more efficient than MY; **S. schenckii** — SAB and Y were better than MY. In the remaining media growth was comparable; **N. asteroides** — No growth was obtained in M5F. Y was also inferior to SAB, M and MY; **A. fumigatus** — M5F and MY were inferior to SAB, M and Y. The latter 3 media produced comparable growth; **C. neoformans** — No growth occurred on MY. Growth was similar in the remaining 3 media but the change in color exhibited by LBS made identification easier.

TABLE I

Microorganisms used to artificially contaminate sputum samples

Microorganism and designation	Isolated from	Isolation date
A. fumigatus		
LR.	Sputum	June, 1978
BC.	Lung biopsy	April, 1978
C. neoformans		
FA.	CSF	January, 1977
CA.	Blood	April, 1978
H. capsulatum		
LEE.	Sputum	December, 1977
C. 307	Lymph node	August, 1978
N. asteroides		
LD.	Sputum	September, 1977
FM.	Sputum	August, 1976
P. brasiliensis		
BA.	Oral mucosa	April, 1978
MTC.	Sputum	June, 1978
S. schenckii		
RF.	Draining sinus	December, 1977
FH.	Skin lesion	August, 1978

TABLE II

Recovery of **H. capsulatum**, **P. brasiliensis**, **S. schenckii** and **N. asteroides** from seeded sputum samples. Effect of digestion and culture media

Digestion Procedure	Culture Medium — Mean number of colonies/plate (*)				
	Sabhi	Modified Sabouraud	Modified Sabouraud 5 fluoro-cytosine	Yeast extract	NH_4OH yeast extract
H. capsulatum					
— Untreated	141.7	59.8	18.8	52.2	52.0
— NAC (**)	124.6	49.3	4.8	41.6	28.4
— Pancreatin	116.3	53.0	4.5	47.5	21.3
P. brasiliensis					
— Untreated	18.0	40.8	NG(***)	40.3	18.2
— NAC (**)	5.5	42.0	NG	24.7	15.2
— Pancreatin	5.7	45.5	NG	30.1	26.6
S. schenckii					
— Untreated	102.5	90.5	79.7	94.2	62.5
— NAC (**)	89.5	71.7	77.3	76.8	42.3
— Pancreatin	103.6	95.0	90.2	93.3	39.8
N. asteroides					
— Untreated	171.5	156.7	NG	43.1	141.0
— NAC (**)	139.7	124.2	NG	72.4	119.3
— Pancreatin	183.4	155.9	NG	62.9	177.1

(*) Figures represent the mean for 17 different experiments
(**) NAC = N'acetyl-1-cystein
(***) NG = no growth

TABLE III

Recovery of **A. fumigatus** and **C. neoformans** from seeded sputum samples. Effect of digestion and culture media

Digestion Procedure	Culture Medium — Number of colonies/plate (*)						
	Sabhi	Modified Sabouraud	Modified Sabouraud 5 fluoro-cytosine	Yeast extract	NH_4OH yeast extract	Sabouraud	Littman bird-seeds
A. fumigatus							
— Untreated	152.4	113.7	40.5	126.6	51.9	148.2	ND (***)
— NAC (***)	190.1	118.6	58.8	128.4	67.3	154.7	ND
— Pancreatin	189.5	115.0	64.6	109.2	39.6	164.7	ND
C. neoformans							
— Untreated	74.9	ND	ND	ND	NG (****)	67.9	135.6
— NAC (**)	97.4	ND	ND	ND	NG	78.3	151.6
— Pancreatin	81.1	ND	ND	ND	NG	83.1	116.2

(*) Figures represent the mean for 17 different experiments
(**) NAC = N'acetyl-l-cystein
(***) ND = Not done
(****) NG = No growth

Table IV also shows that, in every medium, the lower colony counts corresponded to **P. brasiliensis.** Also, it is observed that the presence of certain antibiotics such as chloromycetin (Sab. M) or penicillin-streptomycin (Y), was not deleterious to **N. asteroides** growth. Contrariwise, gentamicin plus 5 fluorocytosine (M5F) inhibited its growth.

TABLE IV

Average colony counts of various microorganisms according to culture medium

Fungus	Culture Medium (Observation) — Mean number of colonies/plate (*)						
	Sabhi (SLB)	Modified Sabouraud (M)	Modified Sabouraud 5-fluoro-cytosine (M5F)	Yeast extract (Y)	NH_4OH yeast extract (MY)	Sabouraud (S)	Littman bird seeds (LBS)
H. capsulatum	127.6	54.0	9.4	47.1	33.9	ND (**)	ND
P. brasiliensis	9.7	42.7	NG (***)	31.7	20.0	ND	ND
S. schenckii	98.5	85.7	82.4	88.1	48.2	ND	ND
N. asteroides	164.8	145.6	NG	59.4	145.8	ND	ND
A. fumigatus	177.3	115.7	54.6	121.4	52.9	155.9	ND
C. neoformans	84.5	ND (°)	ND (°)	ND (°)	NG	76.4	134.4

(*) Figures represent the average colony counts for the 3 digestion procedures used
(**) ND = Not done
(***) NG = No growth
(°) Medium with cycloheximide

The frequency of contamination exhibited by the various media was assessed (Table V). Among the cycloheximide-free media, (Sab. S, LBS, MY) the highest contamination rate corresponded to SAB (28.5%) and the lowest to LBS (1.8%). The former medium was used with all fungi and the latter with **C. neoformans** only. S and MY had intermediate figures, 11.7 and 3.9%, respectively. The 3 media with cycloheximide exhibited similar contamination rates ranging from 11.3 to 16.4%.

TABLE V
Frequency of contamination exhibited by various culture media

Culture Media	No. contaminated plates / Total No. plates	% Contamination
Sabhi	120/421	28.5
Modified Sabouraud	51/310	16.4
Modified Sabouraud 5-Fluorocytosine	45/310	14.5
Yeast extract	35/310	11.3
NH_4OH yeast extract	12/310	3.9
Sabouraud	13/111	11.7
Littman bird seeds	1/ 54	1.8

Time required to produce detectable colonies was also investigated (Table VI). Eighty percent of the **S. schenckii** — and 94.02% of the **C. neoformans** — colonies grew within the first week of incubation. By this time, 62% **A. fumigatus** and 52% of **N. asteroides** cultures were also positive. **H. capsulatum** and **P. brasiliensis** were late comers as only half of the isolates were recovered after 2 weeks. For **P. brasiliensis**, 39% of the isolations were obtained only after 3-4 weeks of incubation.

TABLE VI
Time required for isolation of pulmonary pathogens

Microorganisms	Total number of isolations	Time in Weeks 1		2		3 — 4	
		No.	%	No.	%	No.	%
H. capsulatum	222	49	(22)	120	(54)	53	(24)
P. brasiliensis	253	13	(5)	141	(56)	99	(39)
S. schenckii	231	186	(80)	34	(15)	11	(5)
N. asteroides	192	99	(52)	76	(39)	17	(9)
A. fumigatus	265	165	(62)	57	(22)	43	(16)
C. neoformans	227	213	(94)	13	(5.6)	1	(0.4)

DISCUSSION

Contamination of sputum cultures with resident bacteria and/or airborne fungal spores hinder isolation of the respiratory fungal pathogens. The former type of contamination has been a problem in regions where antibiotics are freely used[9], as this may result either in decreased susceptibility to the antibacterial drugs used in the media or in increased yeast populations. The latter fungi are known to inhibit development of the slow growing fungi[12]. As indicated by other Authors[6,11,13], the use of digestion procedures and/or different culture media results in improved recovery rates. We wanted to know if this would also be the case in our area, where automedication is common.

The data revealed that treatment with the mucolytic reagents N-acetyl-1-cystein and pancreatin, did not increase recovery rates in comparison to the untreated samples. Such procedures were not deletereous to the fungi studied. We believe that the digestion procedure is, however, useful as it turns a mucous sample into a homogeneous product. Our experimental procedure used a pooled, mechanically homogenized sample, this is, an altered sample from the point of view of consistency. Consequently, the value of the digestants cannot be properly assessed, although our results clearly indicate that they are harmless to **P. brasiliensis** as well as to other fungi[6,10,11].

The various culture media had diverse capacities to promote growth of the chosen fungi as follows: Sabhi proved adequate for all fungi, except **P. brasiliensis**. However, it exhibited the highest contamination rate (28.5%), which may have precluded isolation of the slow-growing **P. brasiliensis.** THOMPSON et al.[16] had already noticed that contamination was a handicap of this medium. Modified Sabouraud was similar to Sabhi for **H. capsulatum** but it exhibited less

contamination. It was also better than Sabhi for **P. brasiliensis.** Addition of 5-fluorocytosine to modified Sabouraud resulted in significantly lower recovery rates for the 5 microorganisms. Actually, this medium completely inhibited growth of **P. brasiliensis** and **N. asteroides.** We were thus unable to confirm the findings of GARRISON & GORDON [5] for **H. capsulatum.**

Yeast extract agar was comparable to modified Sabouraud in all cases except for **N. asteroides** that gave diminished colony counts. The former medium had lower contamination (11.3%). In this study yeast extract was not superior to modified Sabouraud for **P. brasiliensis,** as was suggested in an earlier publication [12]. In our hands, the use of NH_4OH in conjunction with yeast extract agar improved the results of the parent medium only in the case of **N. asteroides.** The NH_4OH medium gave significantly lower recovery rates for **P. brasiliensis, A. fumigatus** and did not allow growth of **C. neoformans.** Although we observed restricted contamination (3.9%) with this medium, as originally described [15,16], our findings disagree with such reports as we were unable to obtain growth of **C. neoformans** [16] or to increase the isolation rates for **H. capsulatum** [15].

Littman bird-seeds agar, Sabhi and Sabouraud all proved adequate for **C. neoformans;** however, production of pigmented colonies in the former medium makes recognition easier.

As it concerns time, 50% or more of the isolations corresponding to **S. schenckii, N. asteroides, A. fumigatus** and **C. neoformans** were obtained after 1 week of incubation. **H. capsulatum** and **P. brasiliensis** were exceptions as they required 2 weeks of incubation to produce detectable colonies. In fact, 39% of the **P. brasiliensis** and 24% of **H. capsulatum** isolates required 3-4 weeks. This indicates necessity of prolonged culture observation.

It was interesting to find that **N. asteroides** could grew in the presence of chloromycetin. DOLAN [3] determined that concentrations of 16 μg/ml were non-inhibitory but the culture media we used contained higher concentrations (50-100 μg/ml). Whether this observation applies to all isolates or if it reflects only a particular trend for our 2 isolates, remains to be determined.

It was observed that irrespective of the culture medium, the lowest colony counts corresponded to **P. brasiliensis.** This fact plus the slow growth rate exhibited by this microorganism, clearly indicate how fragil it is. Consequently, extreme care must be taken when handling samples suspected of harboring this fungus.

If one were to choose a minimal number of culture media for isolation of the respiratory pathogens common to South America, modified Sabouraud, yeast extract agar, Littman plus bird seeds and Sabhi would form an adequate battery. One medium (LBS) will detect **C. neoformans** by means of pigmented colonies. Sabhi will produce adequate growth of **A. fumigatus, C. neoformans** and in the abscence of contamination, of **H. capsulatum** and **N. asteroides** as well. Modified Sabouraud and yeast extract agars will allow recovery of the slow growers **H. capsulatum** and **P. brasiliensis** and also, of **N. asteroides, S. schenckii** and **A. fumigatus.** Unluckely, there is no single medium that can be recommended for all of the respiratory mycotic agents. Consequently, the advice offered by the treating physician is needed in order to select the best media.

RESUMO

Recuperação de fungos a partir de amostras de escarro: efeito de meios de cultura e processos de digestão

O efeito dos processos de digestão e de vários meios de cultura no isolamento de agentes micóticos respiratórios a partir de amostras semeadas de escarro foi investigado. Os digestantes (N-acetil-l-cisteína e pancreatina) não afetaram a viabilidade dos microrganismos estudados. Entre os vários meios de cultura utilizados, quatro pareceram constituir uma bateria adequada, a saber: Sabhi, Sabouraud modificado, ágar extrato de levedura e Littman e alpiste (sementes para pássaros). Apesar da elevada incidência de contaminação, Sabhi foi eficaz para todos os fungos estudados, exceto **P. brasiliensis.** Ágar-Sabouraud modificado e ágar extrato de levedura favoreceram crescimento adequado de **H. capsulatum, P. brasiliensis, N. asteroides, A. fumigatus** e **Sp. schenckii.** O ágar extrato de levedura mostrou menor contaminação que o ágar Sabouraud modificado. Meio de Littman e sementes para pássaros permitiram o

pronto reconhecimento de colônias pigmentadas de **C. neoformans**. Nenhum melhoramento em taxas de recuperação foi registrado com outros meios de cultura.

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