1960

Comparison of Two Methods for Recovery of Fungi from Clinical Material

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COMPARISON OF TWO METHODS FOR RECOVERY OF FUNGI FROM CLINICAL MATERIAL

by

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A Thesis Submitted to the Faculty of the Graduate School of Loyola University in Partial Fulfillment of the Requirements for the Degree of Master of Science

June

1960

LIFE

Kenneth J. Smith was born in Mukwonago, Wisconsin January 4, 1920. He graduated from Mukwonago High School, Mukwonago, Wisconsin, May 1939. He attended Beloit College, Beloit, Wisconsin from September, 1939 to June, 1943, at which time he was graduated with the degree of Bachelor of Science.

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ACKNOWLEDGMENTS

With sincere gratitude I am thankful to Dr. Einar Leifson for his stimulation, direction and supervision throughout this work. My appreciation is extended to Dr. MacDonald Fulton for his many helpful suggestions and to Dr. Thomas J. Bird for his encouragement.

I am also thankful to my faithful wife and the many friends and associates who made this opportunity possible through their generous sacrifices.

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Chapter 1

INTRODUCTION

Pulmonary infections may be caused by fungi with symptoms not unlike those of tuberculosis. X-ray pictures of pulmonary mycosis and tuberculosis may be almost identical. To definitely establish the mycotic nature of pulmonary infection, it is necessary to isolate and identify the causative organisms. This study is an attempt to develop a concentration method which is effective for the isolation of both tubercle bacilli and pathogenic fungi.

The number of pathogenic fungus cells present in a clinical specimen may be very few. They may escape detection unless concentrated and cultured on suitable media. The various acid and alkali concentration methods for tubercle bacilli are usually lethal for pathogenic fungi. The enzymes, pepsin and trypsin, will digest sputum and are not as lethal to fungi as the chemical methods. Pepsin-citric acid solution at proper pH is an efficient digestant of sputum. Pentane, after the method outlined by Palen (1956), is an efficient concentrating agent.

STATEMENT OF PROBLEM

1. To study efficieney of various media for recovery of fungi.
2. To review past work along this line.
3. To develop an effective digestant for sputum which is not injurious to fungi or tubercle bacilli.
4. To test the efficiency for fungi of the concentration methods developed for tubercle bacilli.
5. To present comparative data obtained with two methods for the isolation of fungi from sputum.

Chapter II

LITERATURE REVIEW

Digestion and Concentration

Griffith (1914) used 10% sodium hypochlorite which he added in equal proportion to the sputum specimen. He incubated at room temperature for 20 minutes; then centrifuged, decanted, resuspended and recentrifuged the sediment.

Petroff (1919) introduced the use of sodium hydroxide as digestant. He used 3% concentration in 1:1 ratio with sputum. Lurie (1923) used 3% hydrochloric acid and 6% sulfuric acid. In (1929) Corper found 6% oxalic acid more efficient than 6% sulfuric acid. Corper (1946) discarded acid digestants in favor of 10% trisodium phosphate. He found that a greater proportion of tubercle bacilli survived this method than any method he had used previously.

Hosaya and Soeda (1951) digested specimens with detergents. Oxvan (dimethyl-lauryl-benzyl-oxyethyl-ammonium chloride) and Laboran (cetyl-diethyl-methyl-ammonium methosulphate) were used in 0.5% solution up to 24 hours. Good recoveries were obtained with Mycobaterium tuberculosis.

Ajello (1951) studied the effect of various digestion methods on the recovery of fungi from sputum. The following digestion methods were used: 4% sodium hydroxide, 5% sulfuric

acid, and 10% trisodium phosphate. The pooled sputa were first homogenized, and then centrifuged to remove any yeast or yeast-like organisms which might be present. The prepared sputum samples were then seeded with Blastomyces dermatitidis, Candida albicans, Coccidioides immitis, Cryptococcus neoformans, Geotrichum candidum, Histoplasma capsulatum and Nocardia asteroides.

With four of the fungi, B. dermatitidis, C. immitis, G. candidum and H. capsulatum only 25% recovery was obtained. All methods affected these fungi adversely. N. asteroides was recovered in all cases where trisodium phosphate and 4% sodium hydroxide were used as digestants but not with the acid digestant. C. albicans was recovered in each instance only with sulfuric acid and trisodium phosphate. C. neoformans survived the 4% sodium hydroxide concentration method but survived very poorly with the others.

Marwin (1959) found four surface-active agents which increased the growth of the following pathogenic fungi: Blastomyces dermatitidis, Candida albicans, Cryptococcus neoformans, Epidermophyton floccosum, Histoplasma capsulatum, Microsporum Audoini, Sporotrichum Schencki, Trichophyton mentagrophytes, and Trichophyton rubrum. These agents were Nonisol 100 (polyethylene glycol of lauric acid), Polyethylene

glycol 400 monolaurate (monoester of polyethlylene glycol 400), Mulsor (long chain fatty ester with ether linkage) and Pluronic 164 (condensate of ethylene oxide with a hydrophobic base).

Enzyme Digestion

McNamara (1935) added 0.3% pepsin and 0.6% hydrochloric acid to sputum and incubated for 12 hours. He neutralized with N/1 sodium hydroxide and centrifuged for one hour.

Sullivan and Sears (1939) digested 50 ml of sputum with 0.5 gram of papain at room temperature for 10 to 15 minutes. The digest was centrifuged and the sediment treated with 4% sodium hydroxide before cultivation.

Haynes (1942) prepared a digestant of 0.5% trypsin and 0.7 ml of N/1 sodium hydroxide with pH of 8.5. This mixture was filtered and used 1:1 with sputum. The sputum sample and digestant were incubated at 37°C for 30 minutes. It was then centrifuged at 3,000 r.p.m. for five minutes. The sediment was further treated with 5% oxalic acid for guinea pig inoculation.

Vincent and Birge (1947) demonstrated survival for 5 days of tubercle bacilli in gastric contents at neutral pH. This study showed that raising the low pH caused by the hydrochloric acid in gastric contents permitted best recovery. They noted that contaminants were inhibited at pH 6 which did not effect tubercle bacilli.

Rawlins (1953) used trypsin, chymotrypsin, ribonuclease, desoxyribonuclease and amylase each in 1% concentration

at pH 7.6. Each enzyme-sputum mixture was incubated at 37°C until liquifaction of the sputum was complete. This procedure enhanced recovery of various respiratory pathogens.

Palen (1956) used 0.5% pepsin combined with 2% citric acid in a 1:1 ratio with sputum.

Stinson and Fahlberg (1957) used 1% trypsin with equal amounts of sputum and homogenized by manual shaking. These specimens were incubated at 37°C for one hour. This method was used to concentrate tubercle bacilli and fungi. The test organisms were; N. asteroides, C. albicans, C. neoformans, M. tuberculosis, H. capsulatum (yeast phase) and B. dermatitidis (yeast phase). These pathogens were recovered along with normal bacterial flora. Zephiran chloride, G-4 and Ceepryn chloride used to control bacterial growth were equally inhibitory to fungi.

Gianforte, Brown and Burkhart (1959) used proteolytic enzymes to liberate pathogenic organisms from tissue specimens. Enzyme X-108 (isolated from fermentation beer of Condiobolus brefeldianus) was compared with trypsin. The trypsin method was less efficient in the liberation of pathogens than enzyme X-108.

Concentration Method

Reed and Rice (1931) studied the layering of tubercle bacilli in the interface between water and hydrocarbons. Smith (1951) evaluated the efficiency of hydrocarbon flotation methods. Palen (1956) used pentane to inactivate extraneous bacteria. This hydrocarbon also concentrated tubercle bacilli in the interface.

Media Modification

Campbell (1947) studied conditions necessary to revert H. capsulatum from mycelial to yeast phase. He used infusion agar plus 8% rabbit or horse blood. The pH of the media was 7.6 to 7.8. He incubated his cultures at 37°C.

Levine and Ordal (1949) experimented with factors influencing the morphology of B. dermatitidis. They found temperature to be a critical factor. Yeast phase was maintained at 35°C but it reverted to mycelial phase at 31°C. Both phases grew well at pH 5.5 to 8.5.

Levine and Novak (1950) studied the effect of various agents on the growth of B. dermatitidis. They found that both glucose and sodium azide stimulated growth.

Levine and Novak (1950) determined the effect of pH on the respiration of B. dermatitidis. They found the optimum for endogenous respiration and glucose oxidation to be between pH 6 and 8.

Canizares and Shatin (1951) used 2,3,5 triphenyl-tetrazolium chloride to determine enzymatic activity in dermatophytes. In some cases addition of the salt to the media aided in identification and differentiation of the test strains.

Georg, Ajello and Papageorge (1954) used Sabouraud agar containing Cycloheximide, Penicillin and Streptomycin to

inhibit saprophytes and permit growth of pathogenic fungi. C. neoformans, Asp. fumigatus and A. boydii are sensitive to cycloheximide. M. asteroides strains may be sensitive to Penicillin or Streptomycin.

Pine (1955) noted that the agar samples tested contained fatty acids which inhibited growth of H. capsulatum. The stimulating effect of whole blood added to agar was due to the presence of sulfhydryl groups and albumin.

Shapiro, Mullins and Pinkerton (1955) compared Littman's oxgall agar and Sabouraud dextrose agar with cycloheximide added. They found the combinations of media efficient in the inhibition of contaminating bacteria, allowing good growth of fungi.

Pine and Peacock (1958) found the mycelial phase of H. capsulatum to be inhibited in the presence of 1% citric acid at 30 and 37°C. However, the yeast phase was stimulated under these circumstances.

Chapter III

PRELIMINARY INVESTIGATION

The literature review emphasized the toxicity to fungi of the usual concentration procedures. This toxicity is mainly due to the use of strong acids or alkalies. Sputum was the specimen of choice in all preliminary experiments. It is the most common clinical specimen submitted for isolation of tubercle bacilli and systemic fungi. Sputum also presents the same problems which may be inherent in processing other specimens, and special problems which may not be found in other materials.

Concentration Methods

Materials: Pepsin (Difco 1:10,000) 0.5% in 2% citric acid (Seitz filtered) pH 2.3, 3% sodium hydroxide solution, N/2 hydrochloric acid, N/1 potassium hydroxide, 0.04% brom thymol blue. A large quantity of T.B. positive sputum was obtained through the cooperation of Miss Ludmilla Kay, Hektoen Institute, Chicago. The sputa were pooled and refrigerated until sufficient quantity was on hand. The volume of pooled sputum necessary to complete an experiment was removed from the refrigerator. It was then homogenized by shaking in a paint shaker for 10 minutes. The supernatant was used in the concentration tests. Centrifugation removed the fungi which might have complicated the study.

The fungi used were: Blastomyces dermatitidis -- strain 45-1442-58, Candida albicans strain G-71, Cryptococcus neoformans -- strain A-309, Geotrichum candidum -- strain 45-6497-57, Histoplasma capsulatum -- strain 105, Nocardia asteroides -- strain 347-57. These strains were supplied through the courtesy of Dr. Libero Ajello, Communicable Disease Center, Chamblee, Georgia.

The fungi were added to 40 ml samples of sputum prior to treatment, in the condition and volume recommended by Ajello (1951).

B. dermatitidis and H. capsulatum were received in the yeast phase. They were transferred to Nutrient Agar (Difco) with 6% sheep blood added and incubated at 37°C for 5 days. C. albicans, C. neoformans and C. candidum were grown at 37°C for four days on Sabouraud dextrose agar. N. asteroides was grown on Sabouraud dextrose agar at 25°C for 30 days. The growth was scraped off, ground and suspended in physiological saline.

Table 1

COMPOSITION OF SEEDED SPUTA

Fungus	Stock Concentration	Quantity of Stock added per 40 ml of sputum	Final Concentration of Fungus v/v
B. dermatitidis yeast phase	4%	7.0 ml	0.7%
C. albicans yeast phase	3%	1.0 ml	0.075%
C. neoformans	4%	10.0 ml	1.0%
G. candidum yeast phase	0.01%	1.0 ml	0.0025%
H. capsulatum yeast phase	4%	10.0 ml	1.0%
N. asteroides ground mycelium	5%	2.5 ml	0.31%

The percentage of stock concentration was determined in a Hopkins centrifuge tube. This type of inocula and quantities were used to seed sputa throughout the remaining experimentation.

Methods: The 40 ml of seeded sputum was divided equally and placed in each of two 50 ml screw capped centrifuge tubes. An equal volume of 3% NaOH was added to one tube. An equal volume of 0.5% pepsin in 2% citric acid was added to the other. Both tubes were shaken vigorously by hand and incubated for 30 minutes in a water bath at 37°C. After digestion the NaOH

sample was centrifuged at 2,000 r.p.m. for 15 minutes. The supernatant was poured off and 3 drops of 0.04% BTB added to the sediment. This sediment was neutralized with N/2 HCl added drop by drop, producing 1 to 2 ml of neutral suspension. Three drops of brom thymol blue (BTB) were also added to the pepsin digestant which was neutralized by adding N/1 KOH drop by drop. A one inch layer of pentane was added to the neutral suspension. The screw cap was replaced, tightened and the tube shaken. This tube was placed at an angle at room temperature for 15 minutes. The interface was drawn off by reaching through the pentane with a dis-po-pette (Scientific Products Co.) and 1 to 2 ml placed in a sterile test tube. One tenth ml of each of the NaOH and the pepsin-pentane concentrates were added to four screw capped slant tubes of each of the three media used. A similar set of tubes for control was inoculated with the seeded sputum before concentration. All tubes were incubated at 37°C with caps ajar. When the slants were dry, the caps were tightened. All tubes were observed after 7, 14, 21 and 28 days.

The three media used in culturing the

various concentrates were:

a. Lowenstein - Jensen medium (Difco)

b. Petragnani medium (Difco)

c. Sabouraud dextrose agar (BBL)

a and b are the media commonly used for isolation of tubercle bacilli. c is used for isolation of fungi. Tubercle bacilli were cultured on a and b from each experiment attesting to the effectiveness of the concentration techniques employed.

Experiment 1 Temperature Effect

Materials: Seeded sputa, incubators set at 20, 25, 30 and 37°C.

Methods: One tenth ml of concentrate from each method was inoculated on each of 4 Sabouraud dextrose agar slants. A set of 4 tubes plus controls were incubated at 20, 25, 30 and 37°C respectively. Observations were made at 7, 14, 21 and 28 days.

Results: Table 2

Table 2

EFFECT OF INCUBATION TEMPERATURES ON THE RECOVERY OF FUNGI FROM CONCENTRATES ON SABOURAUD AGAR

Fungi		Incubation Temperatures 20°C	25°C	30°C	37°C
C. albicans	P-P*	4/4	4/4	4/4	4/4
	NaOH**	0/4	0/4	0/4	0/4
C. neoformans	P-P*	4/4	4/4	4/4	4/4
	NaOH**	4/4	4/4	3/4	0/4
C. candidum	P-P*	4/4	4/4	4/4	4/4
	NaOH**	4/4	3/4	4/4	4/4
N. asteroides	P-P*	4/4	4/4	4/4	0/4
	NaOH**	4/4	4/4	1/4	0/4
B. dermatitidis	P-P*	0/4	0/4	0/4	0/4
	NaOH**	0/4	0/4	0/4	0/4
H. Capsulatum	P-P*	0/4	0/4	0/4	0/4
	NaOH**	0/4	0/4	0/4	0/4
CONTROLS		24/24	24/24	24/24	24/24

P-P* -- symbol for pepsin-pentane concentration method.

NaOH**-- symbol for sodium hydroxide concentration method.

Discussion: The table shows the proportion of positive tubes after 28 days incubation. B. dermatitidis and H. capsulatum failed to survive the concentration methods. C. albicans did not survive the NaOH

treatment. With the pepsin-pentane (P-P) method, growth was obtained at all incubation temperatures. C. neoformans did not grow at 30 and 37°C after NaOH concentration. Growth was obtained at all temperatures after P-P concentration. N. asteroides grew well after P-P concentration up through 30°C. It grew well up through 25°C after NaOH concentration. This strain grew best at 25°C but would grow at 30°C after P-P concentration. This shows some NaOH toxicity since no growth was obtained at 30°C.

Experiment 2 Effect of pH

Materials: Seeded sputa, Sabouraud dextrose agar slants at pH 5,6,7 and 8, incubators set at 25 and 37°C.

Methods: The media were buffered with phosphates. Beckman model G pH meter was used to check the pH of the media. Brom cresol purple (BCP) indicator was added to media of pH 5 and 6. BTB indicator was added to media of pH 7 and 8. One tenth ml of concentrate from each method was inoculated on each of 4 Sabouraud dextrose agar slants adjusted to proper pH. One tenth ml of stock fungus specimen, before concentration, was added to each of 4 tubes of Sabouraud dextrose agar slants adjusted to proper pH as control. These cultures and controls were

incubated at 37°C except *N. asteroides* which was incubated at 25°C. Readings were made after 28 days.

Results: Table 3

EFFECTS OF pH OF MEDIA ON RECOVERY OF SPECIFIC FUNGI

Fungi	Method	Incubation temperature	pH of media 5	6	7	8
C. albicans	P-P	37°C	4/4	4/4	4/4	4/4
	NaOH		0/4	0/4	0/4	0/4
	Control		4/4	4/4	4/4	4/4
C. neoformans	P-P	37°C	4/4	4/4	4/4	4/4
	NaOH		0/4	0/4	0/4	0/4
	Control		4/4	4/4	4/4	4/4
G. candidum	P-P	37°C	0/4	4/4	4/4	3/4
	NaOH		0/4	0/4	0/4	0/4
	Control		4/4	4/4	4/4	1/4
N. asteroides	P-P	25°C	4/4	4/4	4/4	4/4
	NaOH		4/4	4/4	4/4	4/4
	Control		4/4	4/4	4/4	4/4
B. dermatitidis	P-P	37°C	0/4	0/4	0/4	0/4
	NaOH		0/4	0/4	0/4	0/4
	Control		4/4	4/4	4/4	4/4
H. capsulatum	P-P	37°C	0/4	0/4	0/4	0/4
	NaOH		0/4	0/4	0/4	0/4
	Control		0/4	4/4	4/4	0/4

Discussion: Varying the pH of the medium had some effect on recovery of the test organisms. C. candidum did not grow well in the control medium at pH 8. H. capsulatum did not grow at pH 5 and 8 on the control medium. B. dermatitidis and H. capsulatum did not survive the concentration methods. C. albicans and C. candidum did not survive the NaOH concentration technique. These data show that Sabouraud dextrose agar at pH 6 to 7 was satisfactory for growth of the test fungi. It also shows that pH 5 and pH 8 were harmful to some test organisms.

Hereafter Sabouraud dextrose agar will be used at the manufacturers pH of 5.6.

The following experiments were designed to determine the reason for the failure of B. dermatitidis and H. capsulatum to survive the pepsin-pentane concentration method.

Experiment 3 B. dermatitidis and H. capsulatum exposed to pentane

Materials: Seeded sputa, pentane (Coleman & Bell), Sabouraud dextrose agar plus indicator.

Methods: The proper concentration (Table 1) (P. 14) of the test organisms was diluted 1:1 with 0.85% NaCl. A one inch layer of pentane was added to each tube.

The tubes were shaken at 5 minute intervals during the 30 minutes of incubation at room temperature, approximately 25°C.

Results:

Table 4

GROWTH OF FUNGI AFTER PENTANE TREATMENT

Fungi	Recording periods in days			
	7	14	21	28
B. dermatitidis	3/4	3/4	4/4	4/4
H. capsulatum	0/4	0/4	2/4	2/4
Control	2/2	2/2	2/2	2/2

Discussion: The pentane has some toxic effect. This is especially noted with H. capsulatum. Recovery of 50% or less may be expected if the exposure time is 30 minutes. However, in actual practice, the exposure time should be closer to 15 minutes. This may increase the percentage of recovery.

Experiment 4 Two Fungi Exposed to Citric Acid

Materials: Seeded sputa, 2% citric acid solution

Methods: Tubes of seeded sputa each containing B. dermatitidis and H. capsulatum were mixed with equal volumes of 2% citric acid. The mixtures were incubated at 37°C for 30 minutes in a water bath. The tubes were shaken frequently during

this period. After incubation the mixtures were centrifuged for 15 minutes at 2,000 r.p.m. The supernatant was poured off, indicator added and the sediment neutralised with N/1 KOH. One tenth ml of each sediment was inoculated on each of 4 tubes of Sabouraud dextrose agar slants. These tubes were incubated at 37°C for 28 days.

Results:

Table 5

EFFECT OF CITRIC ACID ON GROWTH OF FUNGI

Fungi	Recording periods in days			
	7	14	21	28
B. dermatitidis	0/4	0/4	0/4	0/4
H. capsulatum	1/4	2/4	2/4	2/4
Controls	8/8	8/8	8/8	8/8

Discussion: The exposure to 2% citric acid for 30 minutes had a toxic effect on the test fungi. Shortening the exposure time and raising the pH may prevent this result.

Experiment 5 Enzyme Toxicity Test

Materials: 0.5% trypsin, seeded sputa

Methods: Equal volumes of 0.5% trypsin (Difco 1:250) at pH 7.2 and seeded sputa were mixed in screw cap 50 ml centrifuge tubes. The specimens were shaken

frequently during the 30 minute digestion period in the water bath at 37°C. The digested specimens were centrifuged 15 minutes at 2,000 r.p.m. and the supernatant poured off. The sediment was resuspended in 2 ml of 0.85% saline solution. One tenth ml of each sediment was pipetted onto each of 4 Sabouraud dextrose agar slants and incubated at 37°C for 28 days. Controls were inoculated before digestion with trypsin.

Results: Table 6

EFFECT OF TRYPSIN DIGESTION ON GROWTH OF FUNGI

Fungi	Recording periods in days			
	7	14	21	28
B. dermatitidis	4/4	4/4	4/4	4/4
H. capsulatum	3/4	4/4	4/4	4/4
Controls	8/8	8/8	8/8	8/8

Discussion: No toxicity was observed due to trypsin digestion.

Experiment 6 Results with Various Media

Materials: Trypsin 0.5%, sputa seeded with B. dermatitidis and H. capsulatum, tubes of Sabouraud dextrose agar containing 0.01%, 0.05%, 0.5% and 1.0% Tween 80, 0.01%, $CaCl_2$ and Sabouraud dextrose agar slants.

Methods: Each sputum was diluted 1:1 with 0.5% trypsin and incubated in a water bath at 37°C for 30 minutes. Controls were inoculated directly from the seeded sputa. After incubation the tubes were centrifuged at 2,000 r.p.m. for 15 minutes. The supernatant was poured off and the sediment suspended in 2 ml of 0.85% NaCl. One tenth ml of each sediment was added to each of 4 tubes of the various media. Slants were incubated at 37°C and read at 7 day intervals for 28 days.

Results:

Table 7

GROWTH OF B. DERMATITIDIS ON VARIOUS MEDIA

Media	Recording periods in days			
	7	14	21	28
Tween 0.01%	4/4	4/4	4/4	4/4
Tween 0.05%	4/4	4/4	4/4	4/4
Tween 0.5%	3/4	3/4	3/4	3/4
Tween 1.0%	1/4	4/4	4/4	4/4
$CaCl_2$ 0.01%	4/4	4/4	4/4	4/4
Controls	4/4	4/4	4/4	4/4

Discussion: The modified media did not measurably change the recovery of this test organism. The addition of 1% Tween 80 seemed to retard initial growth.

Results:

Table 8

GROWTH OF H. CAPSULATUM ON VARIOUS MEDIA

Media	Recording periods in days			
	7	14	21	28
Tween 0.01%	3/4	3/4	4/4	4/4
Tween 0.05%	3/4	4/4	4/4	4/4
Tween 0.5%	4/4	4/4	4/4	4/4
Tween 1.0%	3/4	3/4	4/4	4/4
$CaCl_2$ 0.01%	3/4	3/4	3/4	3/4
Controls	4/4	4/4	4/4	4/4

Discussion: The supplemented media, Tween 0.05 and 0.5% produced the best growth. These results however did not encourage further work along this line.

Experiment 7 Trypsin - Pentane Concentration

Materials: Seeded sputa, trypsin, pH indicator, Sabouraud dextrose agar, Lowenstein-Jensen and Petragnani media.

Methods: Trypsin solution, 0.5%, was added to the seeded sputa in a 1:1 ratio. The tubes were incubated in a water bath at 37°C for 30 minutes with frequent shaking. After digestion, a one inch layer of pentane was added to each tube. The caps were tightened, the tubes were shaken and placed

at an angle at room temperature for 15 minutes. One tenth ml of the interface was added to each of 4 tubes of Sabouraud dextrose agar slants. All tubes and controls were incubated at 37°C. Observations were made at 7, 14, 21 and 28 days.

Results: Table 9

EFFECT OF TRYPSIN DIGESTION AND PENTANE FLOTATION

Fungi	Recording periods in days			
	7	14	21	28
B. dermatitidis	4/4	4/4	4/4	4/4
H. capsulatum	4/4	4/4	4/4	4/4
Controls	3/3	3/3	3/3	3/3

Discussion: The four tubes of Lowenstein-Jensen and four tubes of Petragnani medium were completely digested in every case. The digestion occurred in spite of two washings of the inocula prior to inoculation of the media. The Sabouraud dextrose agar was not affected by the enzyme. This work was performed to detect any toxic effect of combining enzyme and pentane. The table shows no evidence of toxicity from the trypsin-pentane combination.

Experiment 8 Recovery with Digestant at a Higher pH

Materials: Seeded sputa, suitable volumes of 2% citric acid-0.5% pepsin digestant adjusted to pH 3, 3½, 4, 4½, and 5.

Methods: The various digestion solutions were buffered with phosphates. The pHs were measured with the model G Beckman pH meter. Specimens of seeded sputa and the various digestants were mixed 1:1 and incubated at 37°C for 30 minutes. The digested specimens were neutralized and shaken with pentane. A one tenth ml sample of the interface from each digestion tube was inoculated onto groups of 4 tubes of Sabouraud dextrose agar. The cultures and controls were observed at 7, 14, 21 and 28 days. The sputum specimens were satisfactorily digested at all pH values.

Results:

Table 10

RESULTS OF DIGESTION AT VARIOUS pH VALUES

Fungi	ph Values				
	3	3½	4	4½	5
B. dermatitidis	4/4	4/4	4/4	3/4	3/4
H. capsulatum	4/4	4/4	4/4	3/4	4/4
Controls	8/8	8/8	8/8	8/8	8/8

Discussion: The results indicate that up to and including pH 4 good recovery of the tested fungi was obtained. The seeded sputa were completely digested in 30 minutes up through pH 4. Specimens at pH 4½ and 5 needed up to 60 minutes for comparable digestion.

Experiment 9 Pepsin Digestant Buffered With Sodium Citrate

Materials: Seeded sputa, 2% citric acid-pepsin digestant buffered to pH 3, 3½ and 4 with sodium citrate.

Methods: The 2% citric acid-pepsin digestant had pH of 2.3. Varying amounts of sodium citrate were added to obtain pH values of 3, 3½ and 4. pH determinations were made with a Beckman model G pH meter. Sputum specimens were seeded with the test fungi and divided into nine equal portions. These sputa were mixed in a ratio of 1:1 with the various digestants. The tubes were incubated in a water bath at 37°C for 30 minutes with frequent shaking. After digestion the samples were adjusted to pH 7 and shaken with a one inch layer of pentane. The caps were loosened and the interface allowed to form. One tenth ml of interface material was placed on each of 4 Sabouraud dextrose agar slants. All cultures and controls were incubated at 37°C for 28 days.

Results: Table 11

EFFECT OF CITRIC ACID-PEPSIN BUFFERED WITH SODIUM CITRATE

Fungi	Digestant pH values		
	3	3½	4
B. dermatitidis	3/4	4/4	4/4
H. capsulatum	4/4	4/4	4/4
N. asteroides	4/4	4/4	4/4
Controls	12/12	12/12	12/12

Discussion: Raising the pH of the digestant increases the percentage of positive cultures. The highest pH digestant which gave consistantly high cultural growth was selected. This pH value is 4. Two grams of sodium citrate (Mallinckrodt) buffered 2 grams of citric acid digestant to pH 4. This factor also favored selection of this pH digestant due to the simplicity of composition. This result appears to surmount the difficulty in recovering B. dermatitidis and H. capsulatum.

Experiment 10 Clinical Comparison Between Pepsin-pentane and 3% NaOH Concentration Methods

Materials: Pepsin-pentane reagents (Palen 1956), 3% NaOH solution, Brom Thymol Blue indicator, N/2 HCl, Clinical specimens as submitted for T.B. and or

fungus examination.

Methods: All specimens of 20 ml or more requiring digestion were divided equally. One portion was digested with pepsin-citric acid solution at pH 2.3 and the other with sodium hydroxide. The digestion procedures paralleled each other as to time and temperature. Incubation and observation were the same in all cases. One tenth ml portions of concentrate from each method were placed on Lowenstein-Jensen, Petragnani and Sabouraud dextrose media. All tubes were incubated at 37°C and observed for 28 days.

Results: Table 12

RECOVERY OF FUNGI AND TUBERCLE BACILLI FROM SPUTUM BY P-P AND NaOH DIGESTION METHODS

Method	No. of tubes inoculated	No. of spec. submitted	Fungi positive #	Fungi positive %
P-P	450	150	72	48.0
NaOH	450	150	37	24.67

Both methods yielded 1.33% positive cultures for tubercle bacilli.

Discussion: One hundred and fifty specimens were suitable for processing as described above. The pepsin-pentane method yielded almost twice as great a percentage of fungi as the sodium hydroxide method.

Table 13

DATA FROM CLINICAL STUDY

Spec. No.	Type Fungus	Lowenstein-Jensen		Petragnani		Sabouraud Dextrose	
		NaOH	P-F	NaOH	P-F	NaOH	P-F
1	Hormodendrum sp	-	-	-	-	-	+
2		-	-	-	-	-	-
3		-	-	-	-	-	-
4		-	-	-	-	-	-
5	Penicillium sp	-	-	-	+	-	+
	Hormodendrum sp	-	-	-	-	-	-
6	Hormodendrum sp	-	-	-	-	+	-
	Pullularia pullulans	-	-	-	-	-	+
7	Hormodendrum sp	-	-	-	-	-	+
8		-	-	-	-	-	-
9		-	-	-	-	-	-
10	Hormodendrum sp	-	-	-	-	-	+
11		-	-	-	-	-	-
12	Pullularia pullulans	-	-	-	-	-	+
13		-	-	-	-	-	-
14		-	-	-	-	-	-
15	Hormodendrum sp	-	-	-	-	-	+
16	Hormodendrum sp	-	-	-	-	+	-
17	Hormodendrum sp	-	-	-	-	-	+
	C. albicans	-	-	-	-	-	+

DATA FROM CLINICAL STUDY

Spec. No.		Lowenstein-Jensen		Petragnani		Sabouraud Dextrose	
		NaOH	F-F	NaOH	F-F	NaOH	F-F
18	C. albicans	-	-	-	-	+	-
19		-	-	-	-	-	-
20	Hormodendrum	-	-	-	-	-	+
	Penicillium	-	-	-	-	-	+
21	Oospora	-	-	-	-	+	-
	Mucor	-	-	-	-	-	+
22	Hormodendrum	-	-	-	-	-	+
23	Hormodendrum	+	-	-	-	+	+
24	Asp. glaucus	-	-	-	-	-	+
25	Alternaria	-	-	-	+	-	-
	Hormodendrum	-	-	-	-	-	+
26	C. albicans	-	-	-	-	+	-
	Hormodendrum	-	-	-	-	-	+
27	Candida sp	-	-	-	-	+	+
	Hormodendrum	+	-	-	-	-	+
28		-	-	-	-	-	-
29		AFB seen on direct P-P smear only. Cultures neg. for AFB				-	-
30	C. albicans	-	-	-	-	+	-
31	Rhodotorula	-	-	-	-	+	-
	C. albicans	-	-	-	-	-	+

DATA FROM CLINICAL STUDY

Spec. No.	Type Fungus	Lowenstein-Jensen		Petragnani		Sabouraud Dextrose	
		NaOH	F-F	NaOH	F-F	NaOH	F-F
32	Rhodotorula	-	-	-	-	+	-
	C. albicans	-	-	-	-	-	+
33	Rhodotorula	-	-	-	-	-	+
	Mucor	-	-	-	-	+	+
	Asp. fumigatus	-	-	-	-	-	+
	Fusarium	-	-	-	-	-	+
34	Pullularia pullulans	-	+	-	-	-	-
	Aspergillus Niger	-	-	-	-	-	+
35	C. albicans	-	-	-	-	-	+
	Asp. niger	-	-	+	-	-	+
36	C. albicans	-	-	-	-	-	+
37	Chromogens (acid fast)	-	-	+	-	-	-
38		-	-	-	-	-	-
39	Chromogens (acid fast)	-	-	-	+	-	-
40	Chromogens (acid fast)	+	+	+	+	-	-
41	Candida sp	-	-	-	-	-	+
42	Asp. fumigatus	-	+	-	-	-	-
43		-	-	-	-	-	-
44	Hormodendrum	+	-	-	-	+	-
45	Tubercle bacilli	+	-	+	-	-	-
46	C. albicans	-	-	-	-	-	+

DATA FROM CLINICAL STUDY

Spec. No.	Type Fungus	Lowenstein-Jensen		Petragnani		Sabouraud Dextrose	
		NaOH	P-P	NaOH	P-P	NaOH	P-P
47	Candida sp	-	-	-	-	-	+
48	Candida sp	+	-	-	-	+	+
	Hormodendrum	-	-	-	-	-	+
49	Penicillium	-	-	-	-	-	+
	Hormodendrum	-	-	-	-	+	+
50	Hormodendrum	-	-	-	-	-	+
51	Hormodendrum	-	-	-	-	-	+
52		-	-	-	-	-	-
53		-	-	-	-	-	-
54		-	-	-	-	-	-
55	C. albicans	-	-	-	-	-	+
56	C. albicans	-	-	+	-	-	-
	Hormodendrum	-	-	-	-	+	-
57		-	-	-	-	-	-
58		-	-	-	-	-	-
59		-	-	-	-	-	-
60		-	-	-	-	-	-
61		-	-	-	-	-	-
62		-	-	-	-	-	-
63	C. albicans	+	-	-	-	-	-
64		-	-	-	-	-	-

DATA FROM CLINICAL STUDY

Spec. No.	Type Fungus	Lowenstein-Jensen		Petragnani		Sabouraud Dextrose	
		NaOH	P-P	NaOH	P-P	NaOH	P-P
65		-	-	-	-	-	-
66		-	-	-	-	-	-
67		-	-	-	-	-	-
68		-	-	-	-	-	-
69		-	-	-	-	-	-
70		-	-	-	-	-	-
71	Candida sp	+	-	-	-	-	+
72	Candida sp	-	-	-	-	-	+
73	Asp. fumigatus	-	-	-	-	-	+
74	Candida sp	-	-	+	-	-	+
75		-	-	-	-	-	-
76		-	-	-	-	-	-
77		-	-	-	-	-	-
78		-	-	-	-	-	-
79	Candida sp	-	-	+	-	-	+
80		-	-	-	-	-	-
81	C. albicans	-	-	+	-	-	+
82	C. albicans	-	-	+	-	-	-
83		-	-	-	-	-	-
84		-	-	-	-	-	-
85		-	-	-	-	-	-

DATA FROM CLINICAL STUDY

Spec. No.	Type Fungus	Lowenstein-Jensen		Petragnani		Sabouraud Dextrose	
		NaOH	P-P	NaOH	P-P	NaOH	P-P
86	Candida sp	-	-	-	-	-	+
87		-	-	-	-	-	-
88		-	-	-	-	-	-
89	Candida sp	+	-	+	-	-	+
90	Candida sp	+	-	-	-	+	+
91	Candida sp	-	-	-	-	-	+
92	Scopulariopsis	-	-	-	-	-	+
93		-	-	-	-	-	-
94	Helminthosporium	-	-	-	-	-	+
95		-	-	-	-	-	-
96		-	-	-	-	-	-
97	C. albicans	-	-	-	-	-	+
98	C. albicans	-	-	+	-	-	-
99		-	-	-	-	-	-
100		-	-	-	-	-	-
101	C. albicans	-	-	-	-	-	+
102	C. albicans	-	-	-	-	-	+
103		-	-	-	-	-	-
104		-	-	-	-	-	-
105		-	-	-	-	-	-
106	Candida sp	-	-	-	-	+	+
107	C. albicans	+	-	-	-	-	-

DATA FROM CLINICAL STUDY

Spec. No.	Type Fungus	Lowenstein-Jensen		Petragnani		Sabouraud Dextrose	
		NaOH	P-P	NaOH	P-P	NaOH	P-P
108	C. albicans	-	-	-	-	-	+
109		-	-	-	-	-	-
110	C. albicans	-	-	-	-	-	+
111		-	-	-	-	-	-
112	C. albicans	-	-	-	-	-	+
113		-	-	-	-	-	-
114		-	-	-	-	-	-
115	C. albicans	-	-	-	-	-	+
116	C. albicans	-	-	-	-	+	-
117	C. albicans	-	-	-	-	-	+
118	C. albicans	-	-	-	-	-	+
119		-	-	-	-	-	-
120		-	-	-	-	-	-
121	Candida sp	-	-	+	-	-	+
122	Tubercle bacilli	-	-	+	-	-	-
123	C. albicans	-	-	-	-	-	+
124		-	-	-	-	-	-
125	NaOH and P-P direct smears were Positive for AFB. All cultures were negative					-	-
126		-	-	-	-	-	-
127		-	-	-	-	-	-
128		-	-	-	-	-	-

DATA FROM CLINICAL STUDY

Spec. No.	Type Fungus	Lowenstein-Jensen		Petragnani		Sabouraud Dextrose	
		NaOH	F-F	NaOH	F-F	NaOH	F-F
129		-	-	-	-	-	-
130		-	-	-	-	-	-
131	C. albicans	-	-	-	-	+	-
132		-	-	-	-	-	-
133		-	-	-	-	-	-
134		-	-	-	-	-	-
135	Cultures positive for AFB.	-	+	-	+	-	-
136		-	-	-	-	-	-
137	C. albicans	-	-	-	-	-	+
138		-	-	-	-	-	-
139		-	-	-	-	-	-
140		-	-	-	-	-	-
141	C. albicans	-	-	-	-	-	+
142		-	-	-	-	-	-
143		-	-	-	-	-	-
144		-	-	-	-	-	-
145	C. albicans	-	-	-	-	-	+
146	Asp. fumigatus	+	-	-	-	-	-
147	Asp. fumigatus	-	-	-	-	-	+
148		-	-	-	-	-	-

DATA FROM CLINICAL STUDY

Spec. No.	Type Fungus	Lowenstein-Jensen		Petragnani		Sabouraud Dextrose	
		NaOH	P-P	NaOH	P-P	NaOH	P-P
149		-	-	-	-	-	-
150	C. albicans	-	-	-	-	-	+
Total fungi		10	2	7	3	20	67
Total tubercle bacilli		1	1	1	1	-	-
Total A.F. chromogens		1	1	2	2	-	-

Organisms isolated	Fungi	T.B.	Chromogens
NaOH method	37	2	3
P-P method	72	2	3

Table 14

SUMMARY OF DATA FROM CLINICAL STUDY

	Pepsin-pentane method		Sodium hydroxide method	
	No. of strains isolated	Per cent	No. of strains isolated	Per cent
Candida albicans	22	30.8	11	29.2
Hormodendrum sp.	16	22.4	10	28.1
Candida species (other)	13	18.1	11	29.2
Alternaria sp.	1	1.4	--	---
Aspergillus fumigatus	2	2.8	1	2.7
Aspergillus glaucus	2	2.8	--	---
Aspergillus niger	3.	4.2	1	2.7
Fusarium sp.	1	1.4	--	---
Helminthosperium sp.	1	1.4	--	---
Mucor sp.	2	2.9	1	2.7
Oospora sp.	-	---	1	2.7
Penicillium sp.	3	4.2	--	---
Pullularia pullulans	3	4.2	--	---
Rodotorula sp.	2	2.8	1	2.7
Scopulariopsis sp.	1	1.4	--	---
Totals	72	100%	37	100%

Discussion: Comparison of the two concentration methods shows almost twice as many fungi cultured from pepsin as sodium hydroxide. This result stimulated the author to explore the use of the pepsin-pentane method. Some of the organisms identified are considered contaminants by many workers. However, repeated recovery of an organism would strongly suggest pathogenicity when correlated with clinical history.

The Chi Square (X^2) test was applied to determine the significance of the difference between the results of the two concentration methods. Chi Square was calculated by analyzing a four fold table.

STATISTICAL ANALYSIS OF NaOH AND P-P CLINICAL STUDY

Method	Results				Totals
	Positive		Negative		
	Obs.	Exp.	Obs.	Exp.	
NaOH	a 37	(54.5)	b 113	(95.5)	150
P-P	c 72	(54.5)	d 78	(95.5)	150
Totals	109		191		300

(a) = $\frac{150 \times 109}{300}$ = 54.5

(b) = 150 - 54.5 = 95.5

(c) = 109 - 54.5 = 54.5

(d) = 191 - 95.5 = 95.5

$$X^2 = \frac{(ad - bc)^2 \times n}{(a-b)\ (b-d)\ (c-d)\ (a-c)}$$

$$X^2 = \frac{(37 \times 78 - 113 \times 72)^2 \times 300}{(150)\ (191)\ (150)\ (109)}$$

$$X^2 = 17.6$$

The value obtained for Chi Square with one degree of

freedom is 17,6. This is significant at the 0.1% level. These results appear to indicate that the Pepsin-Pentane concentration method will recover a significantly greater number of fungi than the Sodium Hydroxide method.

Chapter IV

GENERAL DISCUSSION

One of the difficulties in recovering viable fungi from clinical specimens stems from the treatment of the sample. The concentration methods used were satisfactory for recovery of viable tubercle bacilli. Dubos (1954) considered that the concentration methods used at that time for recovery of tubercle bacilli prevented growth of many of the organisms. This applies even more drastically to the effect of these concentrating solutions on fungi. Most workers consider fungal growth to be contaminants which must be avoided during their efforts to recover viable tubercle bacilli.

There is an increased recognition of the role of fungi in pulmonary disease. Several tubercle bacillus concentration methods were evaluated as to their effect on survival of selected fungi by Ajello (1954) and Stinson and Fahlberg (1957).

Experimental evidence is presented to show that a modification of an enzyme concentration method will recover viable fungi as well as tubercle bacilli. The ability to recover acid fast organisms by the Pepsin-Pentane method has been established by Palen (1956). It was also noted that tubercle bacilli recovered from the P-P method stained more

intensely than those recovered from the 3% NaOH method. The P-P method does not destroy morphology of the organisms present in direct smear. This may help establish the earliest evidence of mycotic infection.

Various environmental conditions were produced to check their effect on the test fungi. Table 2 (P. 17) shows that incubation temperature had little effect on recovery after enzymatic digestion. Since B. dermatitidis and H. capsulatum did not grow, no effect of temperature was demonstrated for these organisms. The yeast phase is converted to mycelial phase for both fungi at 30°C and lower. This observation was also noted by Levine and Novak (1950), Levine and Ordal (1946) and Campbell (1947). Changing the pH of the media Table 3, (P. 19), demonstrated the optimal growth of the test fungi to be between pH 6 and 7. The greatest effort was expended to detect factors causing toxicity to B. dermatitidis and H. capsulatum. Each part of the P-P method was checked. Table 4 (P. 21) demonstrated some toxicity due to exposure to pentane for 30 minutes. In actual practice the interface development with pentane can be accomplished in 10 to 15 minutes. Limiting the time of exposure to pentane will decrease this toxicity. This was observed in the case of B. dermatitidis and H. capsulatum where the exposure to pentane limited the frequency of recovery to 50% or less. On the other hand

exposure to pentane for only 15 minutes resulted in 50% or more recovery of these fungi, Table 4 (P. 21). Effects of exposure to citric acid, Table 5 (P. 22) showed the greatest toxic effect. The pH of the digestant was raised because of this experiment. A different enzyme, Table 6 (P. 23) was investigated to determine whether a possible toxicity to fungi if any was due to pepsin. Trypsin was selected for this purpose. It was discarded as a digesting agent since in all cases, enough enzyme was transferred with the digestant to digest the T.B. media. However, it may be an excellent digestant for fungus recovery alone. The use of other enzymes which do not have the disadvantages may be considered for future use. Tables 7 (P. 24) and 8 (P. 25) demonstrate the effect of media modification on the growth of fungi. Several investigators, for example, Marwin (1959) determined the beneficial effect of adding detergents, sugars, and albumin to various media. The addition of Tween 80 did not markedly improve results, over those obtained with the controls. No evidence of toxicity due to combination of enzyme and pentane was noted in Table 9 (P. 26). Raising the pH values of the digestant produced favorable results, Tables 10 (P. 27) and 11 (P. 29). Sodium citrate was selected as buffer because of possible ability to induce conversion of diphasic fungi in mycelial phase to yeast phase. Pine and Peacock (1958) observed this phenomenon. Two

grams of sodium citrate buffered two grams of citric acid to pH 4 after addition of 0.5% pepsin. Data from clinical comparison between the Pepsin-Pentane of Palen (1956) and the 3% NaOH methods are shown in Tables 12 (P. 30) and 13 (P. 31). A significantly greater number of fungi (Chi Square = 17.6) were recovered by the P-P method as compared to the 3% NaOH method. Even though many of the fungi recovered were non-pathogens, this result encouraged experimentation. The modified P-P method will recover pathogens as well as nonpathogens. More work must be done from here on to promote growth of pathogenic fungi and control growth of non-pathogenic fungi recovered from clinical specimens. The use of Mycosel agar (BBL) will be helpful in solving this problem. It does have some limitations (P. 9) which were pointed out by Georg, Ajello, and Papageorge (1954) and Shapiro, Mullins and Pinkerton (1956).

An effort was made to speed up detection of growth on Sabouraud dextrose agar by incorporation of 0.05% 2,3,5 triphenyltetrazolium chloride. However, some of the test fungi did not reduce the salt to red formazan. Canizares and Shatin (1951) used TTC in their study of dermatophytes.

To date, no clinical data are available with the Pepsin Citrate Pentane (P-C-P) method.

The Pepsin-Citrate-Pentane Procedure

(in brief)

Materials:

1) Digestant -- 0.5% pepsin in 2% citric acid buffered to pH 4 with 2 grams of sodium citrate, Seitz filtered.
2) Pentane.
3) Digestion tube (50 ml screw capped), dis-po-petto, medicine dropper bulb, N/1 KOH, 0.04% brom thymol blue.

Procedure:

1) Add an equal volume of digestant to the specimen, incubate at 37°C until digestion is complete. Shake if necessary.
2) Add BTB and neutralize acidity with KOH to light blue.
3) Add 4-5 ml of pentane (one inch layer); tighten screw cap and shake vigorously. Loosen cap and allow interface to form. No flame should be nearby. Pentane is very volatile.
4) Aspirate interface for direct smear and culture.

Summary

A procedure has been presented for the recovery of fungi and tubercle bacilli. The organisms are collected from clinical specimens through use of pepsin-citric acid digestant and concentration at an interface by vigorous mixing with pentane. Analysis of data from a comparative study of the pepsin-pentane and 3% NaOH procedures show that the pepsin-pentane procedure gives:

a. a higher percentage of positive fungus cultures

b. comparable recovery of tubercle bacilli

c. best recovery of fungus pathogens.

LIST OF TABLES

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APPROVAL SHEET

The thesis submitted by Kenneth J. Smith has been read and approved by three members of the faculty of the Graduate School and the Stritch School of Medicine, Loyola University.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

May 23, 1960
Date

Evan Ferson
Signature of Advisor