THE USE OF RAT LIVER CELL CULTURES FOR THE ISOLATION OF *HISTOPLASMA CAPSULATUM* DARLING

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ABSTRACT

THE USE OF
RAT LIVER CELL CULTURES FOR THE
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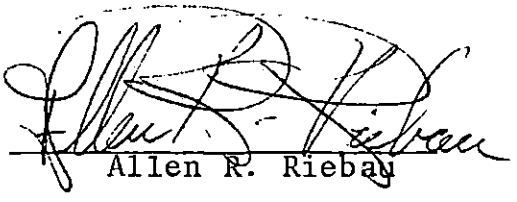
Isolated liver cell cultures were prepared by collagenase digestion, and incubations were maintained in Hans F-12 medium. Active cell cultures were inoculated with aliquots of viable Histoplasma capsulatum suspensions and incubated for varying periods. The cells and medium from the inoculated cultures were separated by centrifugation and plated on Sabouraud dextrose, a support medium to determine the reconversion of viable Histoplasma capsulatum spores or particles. Use of the binomial distribution for evaluation of interaction between liver cells and Histoplasma capsulatum on these plates, showed a positive interaction (99.9% confidence level). Further work in this area may result in a new and universal isolation techniques for the isolation of Histoplasma capsulatum.



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CHAPTER I

INTRODUCTION

Histoplasma capsulatum Darling is a dimorphic deuteromycetic fungus and is the causative agent of histoplasmosis, a pulmonary disease first described by Darling (1908). This disease is characterized in its chronic cavitary pulmonary form by calcified lesions on the upper lobe of the lungs, liver and spleen (Saliba, 1971). It is a widespread disease infecting the population of a number of states, but with a focal point in Kentucky and Tennessee (Edwards, 1969).

One of the major problems involved in histoplasmosis is the paucity of knowledge relating to the ecology of the fungus in native soils (Smith, 1971). Fungal infection occurs from inhaling airborne spores whose production may be enhanced by the presence of bird and bat feces (D'Alessro, *et al*. 1965; Disalwo, 1971). The nutritive factors necessary to support the fungus are unknown (Smith, 1971), but there is a positive correlation between nutritive soil enrichment from guano deposits accumulated below blackbird roosts and the incidence of *H. capsulatum* organisms in the soil (D'Alessro, *et al*. 1965; Disalwo, 1971). One key to epidemiologic understanding of histoplasmosis is the

discovery of a direct culture method for fungal organisms from native soils (Smith, 1971).

The only presently available method for isolating the fungus from soil is the modified oil flotation method designed by Smith and Weeks (1964). This method includes the intraperitoneal injection of soil-derived flotation oil into a host animal. After a suitable period, the animal is sacrificed and the liver and spleen are plated on Sabouraud plates containing dextrose with antibiotics, or as an alternative, yeast extract phosphate media. Slides are also made of tissue section or smears and stained with the methemamine silver technique to observe fungal cells as an intracellular parasite in macrophages or polymorphonuclear leucocytes (Beneke and Rogers, 1970).

The purpose of this study was to investigate the possibility of using isolated rat liver cell cultures for the in vitro isolation of H. capsulatum. Cultures were prepared by methods taken from Seglen (1976) and LaBrecque and Howard (1976). Liver cell cultures were inoculated by a suspension from a culture of H. capsulatum. Later, some cells were removed and plated on Sabouraud plates with dextrose and antibiotics. Observable growth of the typical fungal colonies, as described by Howell (1939), would indicate culture infection by H. capsulatum. Success using this in vitro procedure would eliminate the

need for animal hosts and provide a rapid diagnostic tool for *H. capsulatum*.

CHAPTER II

MATERIALS AND METHODS

Cell Culturing

Liver cell cultures were prepared from adult Sprague-Dawley rats, quartered under a lighting regimen of 12 light: 12 dark. A table sterilizer was used for sterilization of all instruments and containers. Sterilized equipment included a minimum of three pairs of dissecting scissors, three hemostats, several razor blades and a dissection plate (the base plate of a dissection microscope). Ten sterile 2.5 milliliter (ml) syringes plus 15 sterile 20-gauge needles and two 17-gauge needles were also prepared. Siliconized (Siliclad-Clay Adams) sterile glassware, including 30 pasteur pipets and three 25ml erlenmeyer flasks, were prepared. Finally, three or more 50ml flasks were stoppered and sterilized for storage of media, along with four to five screw cap pyrex tubes for use in centrifugation.

Many original procedures were taken from Seglen (1976), Darling (1908) and Bonney, et al. (1971). Care was taken to avoid culture contamination and all glassware and preparation instruments were sterilized. All media was sterile ultracleaned through a millipore filter

(Millipore Co.) 24ml in diameter with a pore size of 0.45μ into stoppered erlenmeyer flasks. A luerloc Millex filter unit (Millipore Co.) was used to aid filtration. All laboratory areas were cleaned with a strong fungicidal disinfectant (Shaklee Corp.) before work began.

Media were normally prepared the day before culturing began. Sixty-five milliliters of Krebs Ringer bicarbonate buffer (Paul, 1975) with 200mg% glucose (KRBG) was prepared, without $MgSO_4 \cdot 7H_2O$ and $CaCl_2$ (Table I). To replace these salts and maintain correct volume, 0.9% NaCl was added in appropriate volumes. To this modified KRBG, 1,000 units of penicillin-G (Sigma Chemical Co.) and 0.5 milligram (mg) per ml of streptomycin sulfate (Nutritional Biochemicals Corp.) were added. This media was bubbled with a gas mixture of 95% oxygen and 5% carbon dioxide and sterile ultracleaned as above. This media served not only to wash the excised liver tissue, as explained by Paul (1975), but also to remove intercellular Ca^{++} and Mg^{++} which inhibit enzymatic cell dispersion, as explained by Seglen (1976).

Table II lists ingredients of the medium. The components and proportions used by Bonney et al. (1971) were modified by the addition of 2.5mg/ml of extra glucose. Antibiotics were added to the final media in

Table I. Initial Medium Constituents (Modified KRBG) for Rat Liver Culture.

Constituent	Stock Solution	Parts in KRBG
NaCl	0.9gm/100ml	100
KCl	0.115gm/10ml	4
KH_2PO_4	0.210gm/10ml	1
*$MgSO_4 \cdot 7H_2O$	0.38gm/10ml	1
$NaHCO_3$	1.294gm/100ml	21
*$CaCl_2$	0.122gm/10ml	3
Glucose	0.002gm/ml of final volume	
**Penicillin-G	1,000 units	
**Streptomycin Sulfate	.5mg/ml	

*Removed from KRBG for this experiment
**Added for this experiment

Table II. Final Medium Constituents for Rat Liver Culture.

Constituent	Percentage or Amount
Fetal Calf Serum	25%
Han's F-12 Medium	75%
Insulin	0.5ug/ml
Glucose	0.0025gm/ml
$NaHCO_3$	add until gassed media buffered to pH 7.2-7.4
*Penicillin-G	50 units
*Streptomycin Sulfate	.025mg

*Added for this experiment in varying quantities

varying amounts. During experimentation, concentrations of 100 units of penicillin-G and 0.05mg of streptomycin sulfate inhibited cell proliferation greatly. A 50% dilution showed no observable inhibition and was adopted for further cultures. After preparation, the final medium was gassed with 95% oxygen and 5% carbon dioxide injected into a 30ml erlenmeyer flask.

A collagenase digestant media normally 3ml was prepared by the addition of lyophilized enzyme (Collagenase-CLS, Worthington Biochemical Corporation) to KRBG without antibiotics to a concentration of 5mg/ml (0.05%) (Seglen, 1976; LaBrecque, 1976). The collagenase media was millipore-filtered into one 25ml sterile, unsiliconized flask.

After preparation, all media were stoppered and stored at $4^{o}C$ until the next day and media over one week old were discarded.

The removal of liver tissue from the rat was modified after the method outlined by Seglen (1976). Each animal was killed under a laboratory hood by cervical dislocation, then quickly secured to a dissection board ventral side up. The abdominal hair was cleaned with 70% ethanol and the skin was cut, with scissors, along the left side, without body cavity penetration. Next the skin was cut transversely across the abdominal

cavity and the rib cage. The skin was then peeled back to reveal the peritoneum and Kimwipes were draped over the animal's body, leaving only the peritoneum exposed. The peritoneum was then swabbed using 70% ethanol to remove any contamination from the animal's coat.

Next the liver was exposed with a second pair of scissors, with hemostats used to hold the tissue away from the exposed liver tissue. With a sterile syringe, cold modified KRBG was injected into one lobe of the liver until the tissue became sandy brown. This indicated that perfusion had taken place (Seglen, 1976). With a third pair of scissors and forceps, this area of the liver was removed and placed on the sterilized dissection plate. A one cubic centimeter (cc) section was finely sliced with a single edged razor blade. With forceps, this section was placed into one of the sterile siliconized flasks and stoppered. A 5cc syringe with a 17-gauge needle was used to introduce 10 ml of the cold KRBG with antibiotics into the flask and to agitate the tissue in this medium for several minutes. This procedure was added to remove intercellular calcium and magnesium and further sterilize the tissue (Seglen, 1976; Paul, 1975).

The modified KRBG was aspirated off and discarded.

An additional 10ml wash was sometimes necessary to remove remaining blood cells. After the final wash, the collagenase digestion media was added, and the flask was incubated in a Dubnoff Metabolic Shaker (Precision Instrument Inc.) at 37°C for 45 minutes at 60 oscillations per minute.

After incubation, the cell suspension was drawn into a syringe and filtered into a 15ml screw top tube through a stainless steel filter base (Luerinlet, Millipore Corp.) with a 133μ pore size filter. The screw top tube was cooled 4°C prior to centrifuging at 1820rpm for three minutes. After centrifugation the supernatant was removed, discarded, and the pellet was resuspended in 5ml of the filtered KRBG. The tube was again cooled and recentrifuged as above. The supernatant was again discarded and the cell pellet was resuspended in the final medium, chilled Hans F-12 (Grand Island Biological Company) and fetal calf serum with insulin, glucose, and antibiotics (see Table II).

Using a siliconized pasteur pipet, the suspension was transferred into 25sq. cm. sterile Falcon Plastics tissue culture flasks (Falcon Plastics). Aliquots from this culture were used to begin new cell cultures. After the cultures were established, they were gassed with a mixture of 95% oxygen and 5% carbon dioxide and incubated

in a Bio-Incubator (Millipore Corporation) at 37°C.

Cell population counts were taken with a Spencer Brightline Hemacytometer (Fisher Scientific Co.) using the trypan blue exclusion test for cell viability. Cultures with 50,000 to 100,000 cells were used for the inoculation experiments.

Treatment of Cultures and Plating

H. capsulatum was secured from a stock culture maintained in the microbiology laboratory at Morehead State University. This culture was a slant-tube culture on Sabouraud's dextrose agar (Pelczar and Reid, 1972; Larsh, 1971). Under a bioflow hood (Fisher Scientific Co.), a small section of the mature fungal culture was removed using a sterilized wire loop. This section (approximately one sq. cm.) was placed into a 25ml stoppered flask containing 10ml of ultrafiltered KRBG. A syringe was inserted through the stopper and the medium was agitated to disperse the fungal pieces and spores. The bottle and syringe were sprayed with a fungicidal disinfectant (Shaklee Corp.) before removal from under the hood.

Fungal suspension aliquots were added to actively growing liver cell cultures in doses of 0.2, 0.3 and 0.5ml and agitated by hand until well mixed with the culture

medium. The inoculated cultures were then incubated for varying durations of time. Medium in these cultures was routinely changed every three days as suggested by Bonney et al. (1971). At each medium change, the medium plus cells were transferred to a 15ml screw top tube and centrifuged at 1820rpm for three minutes. Two culture plates were started at each medium change. One Sabouraud plate was made from 1ml of the supernatant. The other culture plate was started from a two-drop aliquot taken from a resuspension (in fresh KRBG) of the cell pellet. These plates were incubated at room temperature for two to four weeks.

Evaluation of Growth on Plates

Initial plate observations were taken after two weeks of incubation. Plates were observed both macroscopically and microscopically for H. capsulatum. All cultures were categorized as being H. capsulatum growth negative (-), H. capsulatum growth positive (+), or H. capsulatum growth questionable (?). After four weeks, the plates were recategorized as needed.

CHAPTER III

DATA AND RESULTS

The evaluation of H. capsulatum growth in liver cell cultures is presented in Table III. Eleven cultures were derived from cultures started on December 16, 1977. Cultures one through five were inoculated with 0.2ml of the H. capsulatum suspension. Cultures six through nine were inoculated with 0.3ml of the fungal suspension, while culture ten was inoculated with 0.5ml. Culture 11 was used as an untreated control.

Plates numbered 31 through 51 were made from cultures started on January 4, 1978. These cultures received 0.5ml of the fungal suspension. Growth evaluation on these plates appears in Table IV. A tabulation of total plate data is presented in Table V.

Evaluation of cell viability by trypan exclusion demonstrated that 95% of the cultured cells were viable. The results from hemocytometer counts showed that the cells proliferated at a greater rate the first few days after culturing when compared to the proliferation rates later days. The cell proliferation data is shown in Table VI, and the hyperbolic growth curve is illustrated in Figure 1.

Table III. Evaluation of *Histoplasma capsulatum* Plate Cultures (Started December 16).

Sabouraud Plate Number	Liver Cell Culture Number	Source	Evaluation
1	1	supernatant	-
2	1	pellet	-
3	2	pellet	-
4	2	supernatant	-
5	11	pellet	-
6	11	supernatant	-
7	3	supernatant	-
8	3	pellet	-
9	4	pellet	-
10	4	supernatant	-
11	5	pellet	-
12	5	supernatant	-
13	1	pellet	-
14	2	pellet	?
15	3	pellet	-
16	4	pellet	-
17	5	pellet	-
18	9	pellet	+
19	9	supernatant	-
20	10	pellet	-
21	10	supernatant	-
22	6	pellet	-
23	6	supernatant	-
24	7	pellet	+
25	7	supernatant	-
26	8	supernatant	-
27	8	supernatant	-
28	10	pellet	-
29	6	pellet	-
30	3	pellet	-

Table IV. Evaluation of Histoplasma capsulatum Plate Cultures (Started January 4).

Sabouraud Plate Number	Liver Cell Culture Number	Source	Evaluation
31	12	pellet	-
32	13	pellet	-
33	14	pellet	-
34	15	pellet	-
35	16	pellet	-
36	17	pellet	-
37	18	pellet	-
38	12	pellet	-
39	12	supernatant	-
40	13	pellet	-
41	13	supernatant	-
42	14	pellet	?
43	14	supernatant	-
44	15	pellet	-
45	15	supernatant	-
46	16	pellet	-
47	16	supernatant	-
48	17	pellet	-
49	17	supernatant	-
50	18	pellet	-
51	18	supernatant	-

Table V. Plate Data Summary
(December 16 and January 4).

Negative (-)	Positive (+)	Questionable (?)
47	2	2

Table VI. Cell Proliferation Data Summary
(Cultures A and B).

Day	10^4 cells/ml Culture A	% increase	10^4 cells/ml Culture B	% increase
1	7.4	---	5.6	---
2	15.2	105	10.8	92
3	22.4	47	16.0	48
4	24.2	8	18.0	12
5	26.0	7	23.8	32

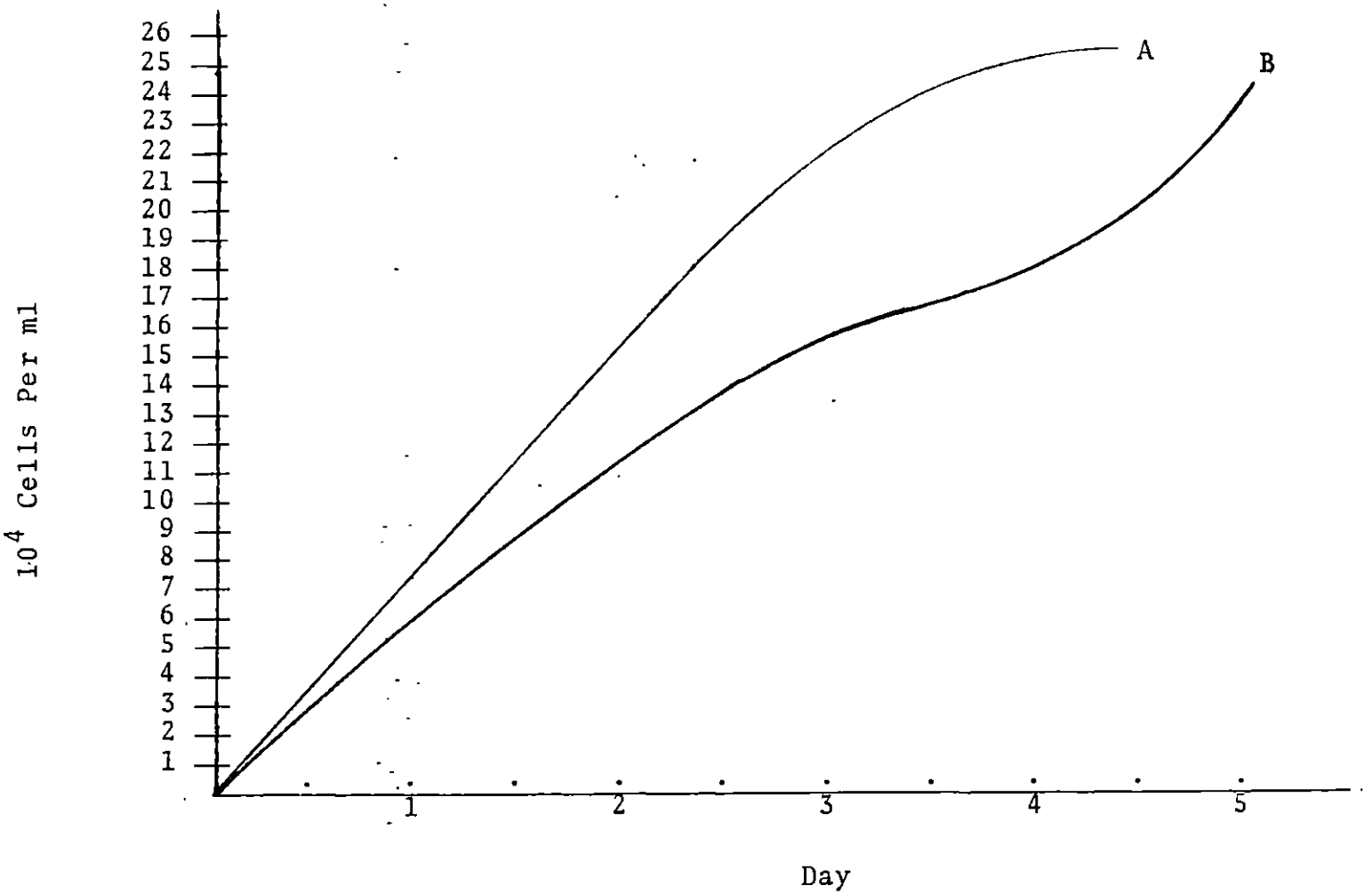

Figure 1. Cell Proliferation Growth Curve (Cultures A and B).

An additional experiment was performed to determine whether the media alone could support H. capsulatum growth. Aliquots of fungal suspension were added to 25sq. cm. falcon tissue culture flasks containing final cell culture media. Each culture was given an exact repetition of treatment to generate plates, except all culture flasks were void of cells. In addition, varying levels of antibiotic load were used in the final media to test for antibiotic inhibition of fungal growth. Data for this experiment is presented in Table VII.

Table VII. Evaluation of Histoplasma capsulatum Growth on Plates Resulting from Suspension of Fungus in F-12 Media.

Sabouraud Plate	Penicillin-G	Streptomycin-Sulfate	ml of Media	Status
H-1	trace	trace	10	+
H-2	trace	trace	10	+
H-3	0.25×10^{-4} gm/ml	1.25×10^{-4} gm/ml	10	+
H-4	0.25×10^{-4} gm/ml	1.25×10^{-4} gm/ml	10	+
H-5	0.505×10^{-4} gm/ml	2.5×10^{-4} gm/ml	10	+
H-6	0.505×10^{-4} gm/ml	2.5×10^{-4} gm/ml	10	+

CHAPTER IV

DISCUSSION

The data gathered from this project is of a categorical type. Plates were evaluated as either positive, negative, or questionable regarding the growth of H. capsulatum fungal colonies. Positive colonies were assesed under conditions given by Howell (1939). Negative plates were evaluated by complete absence of any fungal colonies. Questionable plates were those showing fungal colonies of types which did not measure up to the criteria outlined by Howell (1939).

The binomial test is a common nonparametric tool used in the analysis of categorical data. The bionomial test is simple and usually powerful enough to reject the null hypothesis when it should be rejected. The test statistic in the binomial test has a binomial distribution. If the test statistic (T) equals the number of trials which result in outcome "class 1," where the trials are mutually independent, and where each trial has the probability (p) of resulting in that outcome (as stated by the assumptions), then tne T has the binomial distribution

with parameters p and n (number of trials). The size of the critical region is maximum when p equals the conditional probability, under the null hypothesis. Thus a table of binomial distribution, or the formula $y_r = np + W_r \sqrt{np(1-p)}$, can be used to determine the exact significance level of the test or to define the critical region (Conover, 1971).

The project objective was to demonstrate the feasibility of using liver cell cultures for the isolation of H. capsulatum. This can be stated as a hypothesis of interaction, resulting in growth or no growth on the culture plates. According to scientific convention, the hypothesis of no change or the null hypothesis should be written to state that the probability of interaction is less than or equal to 50% (Ho: $p \leq .50$). The alternate hypothesis, which must express a tendency for uptake of fungal particles and spores by the cultured cells, is stated that the probability of interaction is greater than 50% (Ha: $p > .50$). Then, Ho: $p \leq .50$ and Ha: $p > .50$.

Data statement-

Total number of plates = 51 = n

Total number of negative plates = 47 = T

Total number of positive plates = 2

Total number of questionable plates = 2

Decision rule-

Reject Ho: if $T > t_1$; where t_1 is the high end of the confidence interval as defined by p and the conditional confidence level (α).

$p = .50$ $\quad t_1 = y_r = np + W_r \sqrt{np(1-p)}$

$n = 51$ $\quad$ where W_r is the standard normal random

$\alpha = .999$ $\quad$ variable

$T > t_1$ $\quad t_1 = 51(.5) + 3.902 \sqrt{51(.5)(.5)}$

$47 > 36.53$ $\quad = \underline{36.53}$

Thus, Ho: $p \leq .50$ should be rejected and Ha; $p > .50$ should be accepted with .999 certainty.

With a confidence level of 99.9%, the data gathered from this procedure shows an interaction of the cultured cells inhibiting the growth of fungal colonies on the culture plates. The obvious hypothesis which must now be tested is whether the interactions were due to the interaction of medium alone on the fungal spores and particles. Stated as the hypothesis of no change, or the null hypothesis, the probability of interaction between viable fungal particles and media is greater than or equal to 50% (Ho: $p \geq .50$). The alternate hypothesis is that the interaction between viable fungal particles and medium was less than 50% (Ha: $p < .50$).

Data statement-

Total number of plates = 6 = n

Total number of positive plates = 6 = T

Total number of negative plates = 0

Total number of questionable plates = 0

Decision rule-

Reject Ho: if $T < t_1$; where t_1 is the low end of the confidence interval as defined by p and the conditional confidence level (α).

$p = .50$ $\quad t_1 = y_r = np + W_r \sqrt{np(1-p)}$

$n = 6$ $\quad$ where W_r is the standard normal random

$\alpha = .999$ $\quad$ variable

$T < t_1$ $\quad t_1 = 6(.5) + 3.902 \sqrt{6(.5)(.5)}$

$6 < 7.78$ $\quad = \underline{7.78}$

Thus, Ho: $p \geq .50$ should be rejected and Ha: $p < .50$ should be accepted with .999 certainty.

It can be stated with 99.9% certainty that the proved data does not show a negative interaction of the media on the viability of the fungus under the assumptions of the null hypothesis.

CHAPTER V

CONCLUSIONS

The primary objective of this project was to study the feasibility of using isolated liver cell cultures for the isolation of Histoplasma capsulatum. The criteria used to determine success were that the method of cell culturing must be simple and repeatable, the culture inoculation technique must be repeatable, and a method of demonstrating fungal conversion to the yeast phase in vitro must be devised. The project was ambitious not only because its criteria for success were hard to fulfill, but because there is much controversy among mycologists regarding the validity of even some of the more traditional isolation techniques for H. capsulatum. Although this project may raise more questions than it answers, it has one saving grace: it describes a possible universal isolation technique which could be used in the future to isolate the fungus from soils and hospital specimens. Another possible benefit from an in vitro isolation method could be a means for quantifying fungal particles per unit volume of infected soil. This would be reflected in the actual amount of cellular conversion within the inoculated culture and could be of major importance in studying the ecology of the organism.

This project has not, at this stage, completely fulfilled its criteria for success or demonstrated its potentialities. It has fulfilled certain objectives and indicated ways in which both design and success criteria might be reevaluated. Although the project did not pinpoint a method for direct isolation from either soils or hospital samples, it did demonstrate a positive interaction between viable fungal particles and isolated rat liver cells. However, it did fail to demonstrate the undoubted conversion of H. capsulatum to its yeast phase in vitro.

The low incidence of positive plates indicated a very low probability of any transition to the yeast phase. Those few plates from cell pellets which grew positive fungal colonies could have undoubtedly been due to non-interaction of cells on viable fungal particles. However, cultures do show the possibility of yeast phase conversion in vitro. This is mainly because corresponding plates started from the media above the cell pellets show negative H. capsulatum growth.

Because the data indicated a non-interaction of media on the viability of fungal particles in suspension, some conversion probably occurred.

The factors allowing conversion in such a small percentage of in vitro trials are not defined. If these

factors could be defined and controlled, a new and invaluable research tool might evolve. The isolated liver cell culture offers the possibility of controlling and defining these factors within certain limits because cultures can be manipulated by the investigator. The manipulative range is greatly limited for certain basic parameters in the presently used *in vivo* oil flotation method.

There is a great need for the development of new culturing methods in this area. Any new research should consider the advantages inherent in this experiment. For example, the method of cell isolation and culturing devised here is both simple and dependable. Many cultures can be started from one animal and frozen for later use as defined under normal culturing procedures (Paul, 1975). This could allow for the development of a system that avoids the maintenance expense of a large laboratory animal population. Also, this system would be more responsive than the oil flotation method due to the remission of the incubation period after the injection of flotation oil into the animal (Smith, 1964).

Several major problems do exist, though, in the use of liver cell cultures for the isolation of *H. capsulatum*. One problem is culture contamination by

foreign organisms. Cell culture media are normally very supportive of microorganismic growth, and the addition of a soil derivative containing H. capsulatum would harbor many other microflora and fauna. A method must be devised to allow the cultures to overcome all contaminants, save H. capsulatum. Two possible solutions to this dilemma are the addition of antibiotics in very high concentrations to the soil-derived flotation oil and the heating of cell suspensions to decrease bacterial levels (Paul, 1975).

Another very obvious problem is the lack of a completely demonstrative means for discerning fungus conversion in vitro. One possible answer is culture separation into macrophages and parenchymal cell types, prior to devising a direct intercellular observation method. Again, from evaluation of the data, it appeared that if conversion took place, the fungus was destroyed in almost all cases before it was plated on the agar medium. Because certain plates indicated in vitro conversion success, and because the subsequent media plate reconversion was apparent, the research objectives are still within the realm of possible achievement.

The use of in vitro H. capsulatum conversion as a research tool has great possibilities. Its development depends on serious investigation into the interactions

between cultured cells, either macrophages or parenchymal, and fungal organisms.

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LITERATURE CITED

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APPENDIX

APPENDIX

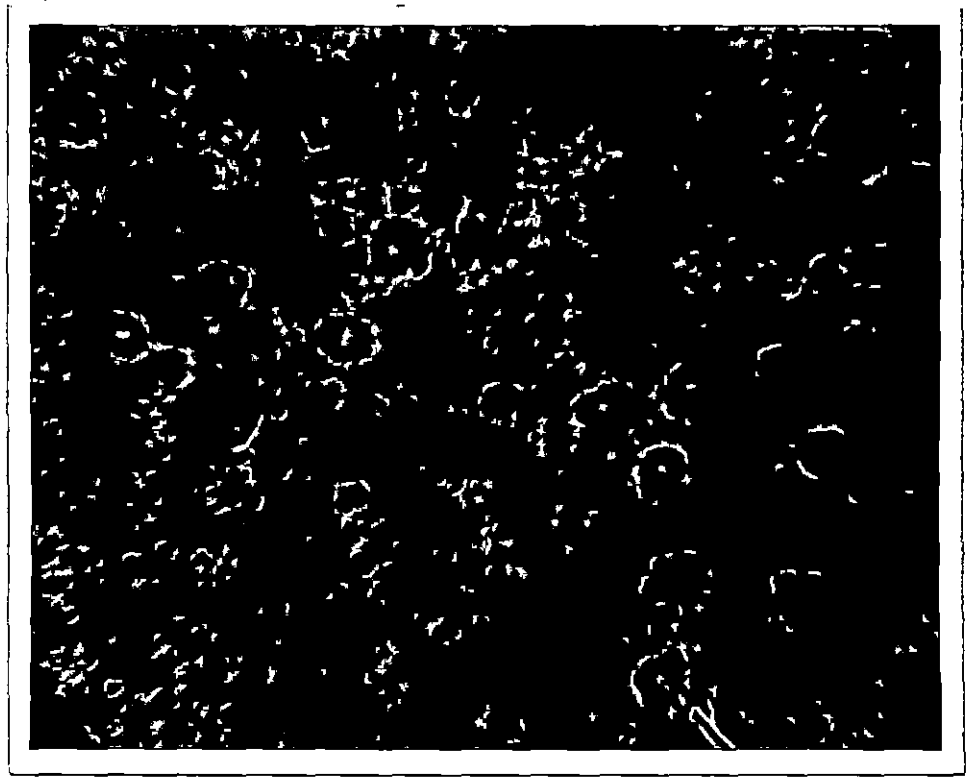

Figure 2. Isolated Liver Cells in Culture

Appendix (Continued)

Figure 3. Fungal Plate Growth

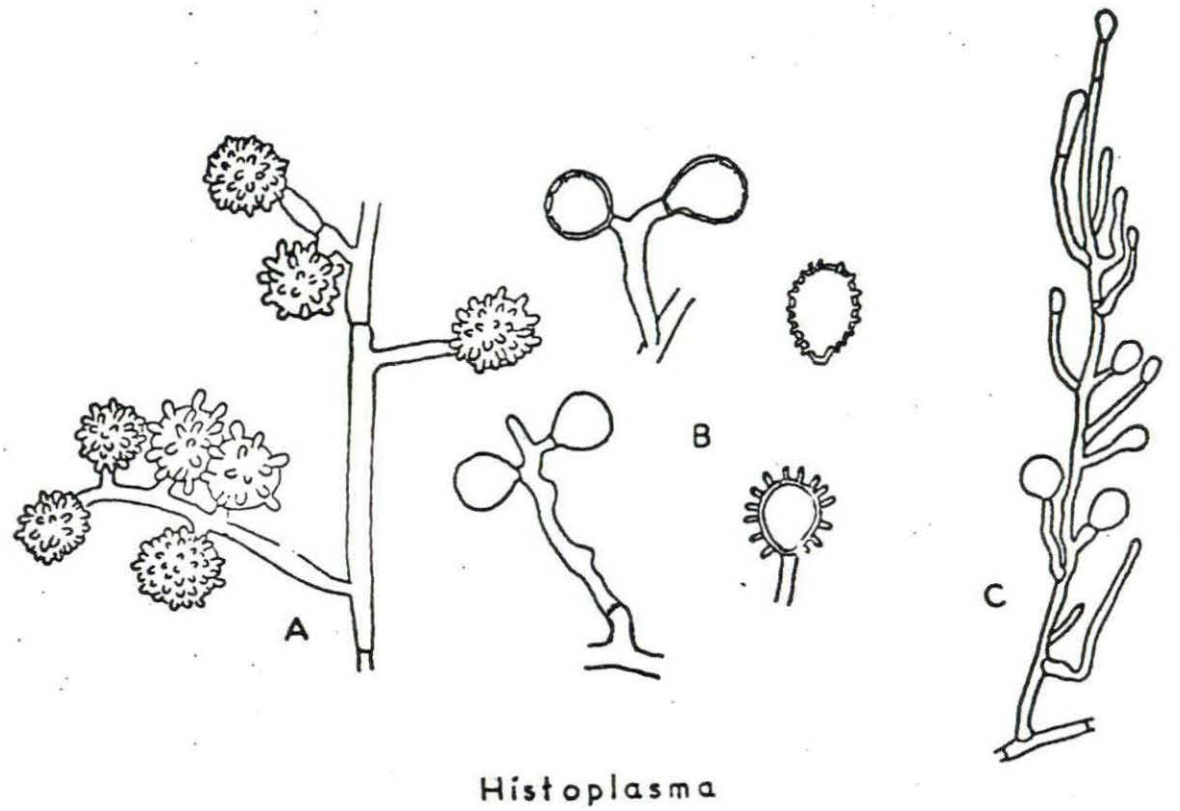

Figure 4. Histoplasma capsulatum. A, hyphae and tuberculate chlamydospores; B, stages in the development of tuberculate chlamydospores; C, smooth-walled chlamydospores developed below the surface of the agar. Redrawn from Howell, Jr., A., 1939. Studies on Histoplasma capsulatum and similar form species. I. Morphology and development.