THE ISOLATION OF HISTOPLASMA CAPSULATUM FROM TISSUES OF EXPERIMENTALLY INFECTED MICE1, 2

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Received for publication June 22, 1951

In the laboratory diagnosis of human histoplasmosis and in the research conducted with animals experimentally infected with Histoplasma capsulatum, a variety of methods have been used for the isolation of this fungus. Thus in the laboratory diagnosis of human infections, Pinkerton (1949) in his review recommends direct microscopic examination of stained preparations of biopsied material such as enlarged superficial lymph nodes, cutaneous, muco-cutaneous or naso-oral lesions, aspiration biopsy of the liver, bone marrow, and blood smears. He also suggests that such biopsied material, as well as stools and sputum, should be cultured. The media that Pinkerton recommends are Sabouraud's agar and blood agar, but he also states that the organism grows readily on many artificial media, including Sabouraud's maltose agar, dextrose blood agar, and potato-dextrose medium. The yeast-like phase of the fungus will grow at 37 C in cultures with a high protein content, such as blood or serum agar. The mycelial phase of the fungus will occur when the cultures are incubated at room temperature. The structures that characterize H. capsulatum, namely, the large, tuberculate chlamydospores, occur only in the mycelial phase.

In spite of a considerable number of clinical reports and experimental investigations, relatively little has been done to evaluate the various technics and media used in the diagnosis of histoplasmosis and the isolation of H. capsulatum from infected tissues. Howell (1948) has reported on the efficiency of two media (brain-heart infusion blood agar and potato-dextrose agar) when using the spleens of infected guinea pigs. He did not, however, correlate his cultural findings with direct microscopic observation of the fungus in the tissues.

This investigation was conducted with the thought of obtaining data on the following points relative to the isolation of H. capsulatum from infected tissues:

1. To determine which tissue (liver, spleen, and heart blood) was most frequently infected with the fungus, both by direct microscopic examination and by cultural examination.

2. To compare the relative efficiency of direct microscopic examination with that of cultural examination.

3. To determine which one of five media (modified Sabouraud's dextrose agar, brain-heart infusion blood agar, brain-heart infusion agar, mycophil agar, and potato-dextrose agar) was most satisfactory for the isolation of H. capsulatum from infected tissue.

1 This investigation was supported by the Medical Research Fund of the Commonwealth of Kentucky.

2 Read before the Laboratory Section of the Southern Branch, American Public Health Association, April 27, 1951, Biloxi, Mississippi.
MATERIALS AND METHODS

Forty-two white mice (undetermined strain) weighing 15 to 20 grams were inoculated intraperitoneally with 0.5 ml of a saline suspension of ground, mycelial phase of *Histoplasma capsulatum*, Sallee strain. (This strain was isolated by one of us from a child in Louisville, Kentucky, and had been under cultivation for about two years.) The inoculum was prepared by grinding four to six weeks' old mycelial cultures in sterile saline with a Ten Brock Pyrex tissue grinder. The mycelial suspensions were adjusted to the density of a no. IV McFarland barium sulfate standard. The mice were autopsied at intervals varying from 16 to 42 days after inoculation. Upon autopsy, the following procedures were conducted on each mouse:

1. Touch tissue impressions were made of the liver and spleen on a clean microscope slide. After drying, the impressions were stained with Leishman's stain. Varying periods of time, up to 45 minutes, were used in making a microscopic examination for the oval, yeast-like, tissue phase of the organism.

2. A blood smear was made of the heart blood, stained with Leishman's stain, and examined in the same manner as in no. 1.

3. Portions of each spleen and liver (approximately 0.25 by 0.25 inches square) were streaked out on a tube (20 by 125 mm) of each of the following media (5 to 10 ml):

   - Modified Sabouraud's dextrose agar, pH 5.6
   - Brain-heart infusion blood agar, pH 7.4
   - Brain-heart infusion agar, pH 7.4
   - Mycophil agar, pH 7.0 (Baltimore Biological)
   - Potato-dextrose agar, pH 5.6

4. About 0.5 ml of heart blood was inoculated into the media mentioned in no. 3.

5. All cultures were incubated at room temperature for one month before being discarded as negative.

All cultures showing a white to tan, cottony mycelium were examined microscopically for the tuberculate chlamydospores characteristic of *H. capsulatum* by making slide preparations with lactophenol-cotton blue. Howell (1948) has shown that cultures on brain-heart infusion blood agar do not show these characteristic structures; consequently, it is necessary to subculture on potato-dextrose agar (or Sabouraud's) since the tuberculate chlamydospores do appear in colonies on these media.

RESULTS

The results of this series of studies are tabulated in the accompanying tables. From table 1 it is evident that:

1. Organisms consistent with the morphology of *H. capsulatum* in the yeast-like, tissue phase were observed in stained tissue impressions (either liver, spleen, or blood) in 36 of 42 (85.7 per cent) infected mice.
### TABLE 1

*Recovery of Histoplasma capsulatum from 42 experimentally infected white mice*

<table>
<thead>
<tr>
<th>NO. OF ANIMALS FOUND INFECTED BY ONE OR MORE POSITIVE CULTURES</th>
<th>NO. POSITIVE</th>
<th>PER CENT POSITIVE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>42</td>
<td>100.0</td>
</tr>
<tr>
<td>NO. OF ANIMALS FOUND INFECTED BY ONE OR MORE POSITIVE TISSUE IMPRESSIONS</td>
<td>36</td>
<td>85.7</td>
</tr>
</tbody>
</table>

* Ground mycelial phase of *H. capsulatum* suspended in sterile saline was injected intraperitoneally. Autopsies were made at intervals ranging from 16 to 42 days after injection.

### TABLE 2

*Cultural recovery of Histoplasma capsulatum from 42 experimentally infected white mice*

<table>
<thead>
<tr>
<th>MEDIUM</th>
<th>NO. CULTURES</th>
<th>NO. POSITIVE</th>
<th>PER CENT POSITIVE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sabouraud's dextrose agar, pH 5.6...</td>
<td>126</td>
<td>111</td>
<td>88.0</td>
</tr>
<tr>
<td>Brain-heart infusion blood agar, pH 7.4</td>
<td>126</td>
<td>113</td>
<td>89.6</td>
</tr>
<tr>
<td>Brain-heart infusion agar, pH 7.4...</td>
<td>102</td>
<td>84</td>
<td>82.3</td>
</tr>
<tr>
<td>Mycoophil agar, pH 7.0</td>
<td>126</td>
<td>92</td>
<td>73.0</td>
</tr>
<tr>
<td>Potato-dextrose agar, pH 5.6</td>
<td>126</td>
<td>101</td>
<td>80.1</td>
</tr>
</tbody>
</table>

* The liver, spleen, and heart blood of each of the 42 mice were inoculated into individual tubes for each medium, except in the instance of brain-heart infusion agar in which only 34 mice were used.

### TABLE 3

*Recovery of Histoplasma capsulatum from experimentally infected white mice*  
by specific tissues and media

<table>
<thead>
<tr>
<th>TOTAL INFECTED</th>
<th>MICROSCOPIC DEMONSTRATION AT ROOM TEMPERATURE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>42</td>
<td>33</td>
</tr>
</tbody>
</table>

Per cent recovered: 78.5, 78.5, 11.9, 80.9, 85.7, 82.4, 64.4, 85.7, 97.6, 65.2, 85.3, 66.7, 80.9, 88.1, 76.6, 88.1, 88.7.

* Ground mycelial phase of *H. capsulatum* suspended in sterile saline was injected intraperitoneally. Autopsies were made at intervals ranging from 16 to 42 days after injection.

BHIBA = Brain-heart infusion blood agar, pH 7.4
BHIA = Brain-heart infusion agar, pH 7.4 (series based on only 34 animals)
MA = Mycoophil agar (Baltimore Biological), pH 7.0
FDA = Potato-dextrose agar, pH 5.6

(2) In each one of the 42 inoculated animals, a positive culture was obtained with either one or more of the tissues and media, giving 100 per cent infection.

(3) In the five different media studies, one tube of each medium was inocu-
lated with a specimen of each one of the three different tissues. The results in table 2 were obtained in regard to positive cultures. On the basis of these results, brain-heart infusion blood agar was the most efficient, although Sabouraud's dextrose agar was only slightly less so.

(4) In table 3 are given the data on the recovery of *H. capsulatum* in these experimentally infected mice showing in greater detail the results broken down by tissues, and by tissue impressions and cultivation on media.

The following results are evident: (a) Liver or splenic tissue impressions had the yeast-like organisms in 36 of 42 (or 85.7 per cent) of the infected mice, while blood smears showed the organism only in 5 of 42 (or 11.9 per cent) of the same series of animals. (b) The highest percentage of positive cultures was obtained with the inoculation of spleen on Sabouraud's dextrose agar, 97.6 per cent. (Actually, the one failure in the splenic culture on Sabouraud's had a contaminant in it. This contaminant may have suppressed the growth of *H. capsulatum*. If this had not happened, a 100 per cent recovery may have been obtained with spleen on Sabouraud's medium.) The other implantations of tissue on culture media gave fewer positive results. The interesting fact to note is the high percentage of positive cultures from heart blood specimens, the same specimens in which such poor results were obtained by microscopic examination of blood smears.

(5) Data were obtained on the correlation of positive cultures and positive stained tissue impressions for each of the three tissues. These data revealed that in all instances less than 85 per cent of the impressions of tissues that were positive by culture were also positive by direct microscopic demonstration of the parasite. Other data also showed that in a high percentage of instances (73.8 per cent) the *Histoplasma* were demonstrable microscopically in both liver and spleen in the same mouse.

In no instance were the organisms demonstrable microscopically in blood smears alone, or in blood smear and liver or spleen (table 4).

**TABLE 4**

*Microscopic demonstration of Histoplasma capsulatum by stained smear technic*

<table>
<thead>
<tr>
<th>TISSUE</th>
<th>RESULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>Spleen</td>
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<tr>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>-</td>
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</table>
DISCUSSION

There has been little systematic experimental work published on the evaluation of various technics and media for the demonstration and isolation of H. capsulatum from the infected host. The vast majority of clinical papers has reported the examination and cultivation of a variety of tissues in trying to recover this infectious agent. Usually, the tissues examined are those which show evidence of pathology. Thus enlarged lymph nodes have been biopsied. Liver tissue obtained by needle biopsy, when the organ was enlarged, has been examined. Bone marrow has been aspirated for examination. Cutaneous and muco-cutaneous lesions have been biopsied when this fungus has been suspected as the etiological agent. The logic of such procedures is based on two facts, (1) the obvious pathological state of the tissue by clinical observation, and (2) the fact that studies of the pathology of proven infections have shown that the reticulo-endothelial tissues are conspicuously parasitized (Pinkerton, 1949).

Levy (1945) presents a table summarizing 55 case reports of proven human histoplasmosis which shows that the three most frequently involved organs were the lungs, liver, and spleen in that order. In a series of experimental infections using 135 white mice infected intravenously with a mixture of yeast-like and mycelial forms (the density of the suspension corresponding to a no. IV McFarland barium sulfate standard), he was able to obtain positive cultures from the livers and spleens of all mice (100 per cent), though the specific medium used was not stated. Levy made a microscopic study of tissue sections of the spleens, livers, lungs, and other tissues of these mice, but does not state what percentage of the mice was positive by this technic.

Parsons (1942) also used the white mouse in producing experimental histoplasmosis by intravenous and intraperitoneal inoculation of the yeast-phase and of the mycelial phase of H. capsulatum. While he states that he obtained a high percentage of infection and death in his experimental animals, he does not indicate in most instances the technic or culture media used in proving his successful infections.

Howell (1948) has reported on the efficiency of methods for the isolation of H. capsulatum. His observations are limited, however, to the culture of a portion of the spleen of infected guinea pigs on each of two plates of brain-heart infusion blood agar and two of potato-dextrose agar. His report does not contain data on the direct microscopic examination of the spleen (either by stained tissue impressions or tissue sections), or data on the value of examination of the liver, blood, or other tissues by either microscopic or cultural technics, or the value of Sabouraud's dextrose agar, a commonly used medium in work on the fungi.

The benefits derived from a systematic evaluation of the efficiency of various tissues and media used in the isolation of H. capsulatum are chiefly of two kinds. One, information gained from such a study would help in concentrating the search for this organism to certain tissues by specific technics. Such data would be useful in the laboratory diagnosis of clinically suspected cases in which the pathology was not obvious. Two, this information could be used in examining
experimentally infected animals for the fungus in chemotherapeutic and other studies where eradication of the infection is attempted.

The results reported in this investigation corroborate the findings of other investigators (Howell, 1948; Levy, 1945; and Parsons, 1942) to mention only a few, that *H. capsulatum* can be recovered in a very high percentage of experimentally infected animals by culture of portions of the spleen and liver. The relatively high percentage of recovery obtained by culture of heart blood was somewhat surprising inasmuch as it has been stated that the organism does not usually appear in the blood (at least, peripheral blood) of human beings, until the terminal stages of the disease. The successful recovery of this fungus in the heart blood of over 75 per cent of the cultures in a group of four different media suggests the value of routine culture of blood from suspected human cases.

The low percentage of recoveries by microscopic examination of blood smears indicates that this technic is a very poor one, especially in light of the relatively high percentage of positive blood cultures. This indicates that while the fungi are in the blood (as shown by positive cultures), they are too few in number on a routine blood smear to be readily found. A thick blood smear or the buffy-coat layer of centrifuged blood in which the leucocytes are concentrated has been used in greatly facilitating the direct microscopic demonstration of this parasite (Lehman, 1950).

The microscopic demonstration of *H. capsulatum* by touch tissue impressions of the liver and spleen in a little over 85 per cent of the cases indicates this examination of these tissues at autopsy or by needle biopsy of the liver to be a highly rewarding technic and has the advantage of being much simpler and more economical, both as to time and materials, than sectioning tissue. Any one of the Romanowsky stains (e.g., Wright, Giemsa, or Leishman) is suitable for staining these tissue impressions.

There appears to be little difference in the incidence of infection in the liver and spleen when using the tissue impression technic in the examination for *H. capsulatum*. This fact is demonstrated both by the percentage of positive tissue impressions for each tissue (table 3) and by the percentage of positive tissue impressions for each tissue when correlated with positive cultural findings for each tissue on the five different media. The variations that do occur in the latter set of data are probably not significant. Levy (1945) appears to have obtained similar results, though this is not clear in his paper.

The grouped data in table 2 show that the highest percentages of positive isolations were obtained with brain-heart infusion blood agar (89.6 per cent), with Sabouraud's (88.0 per cent), brain-heart infusion agar (82.3 per cent), potato-dextrose agar (80.1 per cent), and mycophil agar (73.0 per cent) following in that order. While a slightly higher percentage of recoveries was obtained with brain-heart infusion blood agar, the complexity of the medium and the fact that the characteristic tuberculare chlamydocores of *H. capsulatum* do not appear on this medium, render it probably of very little, if any more, value than Sabouraud's dextrose agar in a practical situation. Indeed, in table 3, where the media are evaluated by the percentage of recoveries for each tissue,
the highest percentage (97.6 per cent) occurred when splenic tissue was cultured on Sabouraud's medium.

These data on the relative efficiency of various media corroborate to a certain extent the findings of Howell (1948), namely, that brain-heart infusion blood agar is the most efficient medium. He was able to obtain 80.4 per cent positive cultures with this medium from guinea pig splenic tissue. We obtained 95.2 per cent positive cultures with brain-heart infusion blood agar and mouse splenic tissue. When mouse splenic tissue was cultured on Sabouraud's dextrose agar, 97.6 per cent recoveries were obtained.

SUMMARY AND CONCLUSIONS

A series of data is presented evaluating the efficiency of two basic technics for the demonstration of Histoplasma capsulatum in 42 experimentally infected white mice. The technics evaluated are Leishman stained tissue impressions of liver, spleen, and heart blood, and the culture of these same tissues at room temperature on five different media: a modified Sabouraud's dextrose agar, brain-heart infusion blood agar, brain-heart infusion agar, mycophil agar, and potato-dextrose agar.

This series of data shows that:

(1) H. capsulatum was demonstrable culturally in 100 per cent of a series of experimentally infected mice by the simultaneous use of five different media.

(2) H. capsulatum was observed by direct microscopic examination of the combined stained tissue impressions of liver, spleen, and heart blood in 85.7 per cent of the experimentally infected mice. The fungus was observed with equal frequency (78.5 per cent) in the liver and spleen by direct microscopic examination of stained tissue impressions. Examination of thin smears of heart blood, however, revealed Histoplasma in only 11.9 per cent of the total number of infected animals.

(3) The highest percentage of cultural isolations of H. capsulatum was obtained on brain-heart infusion blood agar when the positive cultures from the three tissues were combined. The efficiency of cultural isolation for the other four media was in the order given below, with the least efficient one last:

- Sabouraud's dextrose agar
- Brain-heart infusion agar
- Potato-dextrose agar
- Mycophil agar

(4) The highest percentage of cultural isolations of H. capsulatum was obtained with spleen inoculated on Sabouraud's dextrose agar, 97.6 per cent. The other tissues and media combinations gave fairly high percentages of isolations. Except for spleen on brain-heart infusion blood agar, no other combination approximated this high percentage.

(5) A high degree of simultaneous infection was shown to exist between livers and spleens from the same infected animals by demonstration of yeast phase of H. capsulatum in stained tissue impressions and by culture on the media of this study.
REFERENCES


