Mycopathologia et Mycologia applicata, vol. 41, 1–2, pag. 155–166, 1970.

ISOLATION AND IDENTIFICATION OF COCCIDIOIDES IMMITIS FROM NATURAL SOURCES

by

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ABSTRACT

The review of media and techniques that have been developed to date appears to provide more than adequate choice for investigators in endemic areas to perform ecological studies of this organism. The final identification of this organism still lies in the demonstration of its *in vivo* morphology.

Isolation procedures for *C. immitis* from nature have been developed using both *in vivo* and *in vitro* approaches. Since the first *in vitro* soil isolation in 1932 using parasitological techniques on simple media, a variety of spore harvesting procedures and media have been employed. To date, the use of non-peptone media has proven more satisfactory for soil isolation. Yeast extract has proven to be the most satisfactory carbon-nitrogen source. The use of bacterial and anti-fungal antibiotics in media has in essence allowed for the development of useful media. The guinea pig and the mouse have been used as direct differential media in soil isolation. The guinea pig appears the most reliable. Comparison of the three *in vitro* and the mouse *in vivo* techniques show that the double pour and spray procedures to be superior and yield similar results. However, the double pour procedure is favored for general use because of the larger volume of soil samples which can be processed and the more effective safety features of the procedure. None of the four techniques was found to be 100 % efficient using naturally infected soils with low viable counts.

After OPHULS & MOFFITT (19) established in 1900 that "Coccidioidal Granuloma" was caused by a fungus rather than by a protozoan, the question was raised as to where the organism might be found in nature. Although the suspicion existed that the source of the infective agent was probably the soil, workers repeatedly failed to demonstrate its presence using conventional bacteriological techniques. The fungus, *Coccidioides immitis*, was often overgrown by bacteria and other rapid growing fungi among the Phycomycetes.

Paper read at the Eighth International Congresses for Tropical Medicine and Malaria, September 1968, Teheran (Iran).

In his epidemiological review on "Coccidioidal Granuloma" in 1931, BECK (1) postulated that *C. immitis* was a soil saprophyte.

In 1932 STEWART & MEYER (22) demonstrated that *C. immitis* obtained its carbon and nitrogen requirements from very simple sources. They formulated a medium on which *C. immitis* and a few other fungi would grow but which suppressed the growth of most bacteria. They also observed that *C. immitis* was able to survive exposure to a 30—35 % brine for more than 3—4 hours. Using a brine flotation procedure similar to that used in protozoology, they were able to concentrate the spores from soil collected on a ranch near Delano, California, in the San Joaquin Valley. The spore concentrate was pipetted onto the surface of their medium. Thus, the first *in vitro* isolation of *C. immitis* was made.

In 1941 EMMONS (5, 6) isolated *C. immitis* from the soil by inoculating guinea pigs with the concentrated spores (using the STEWART & MEYER spore harvest method) from soil from three widely separated areas in Arizona. In this procedure the guinea pig became the differential medium and simultaneously allowed for the proof of KOCH's postulates. EMMONS refined this animal isolation technique in 1949 and used it to test soil for the presence of *Histoplasma capsulatum* (7). Other workers have used EMMONS' animal method, with and without modifications, to isolate *C. immitis* from soil.

DAVIS *et al.* (2), in 1942 reported the repeated isolation of *C. immitis* from the soil of Panoche Valley in San Bonito County, California. The area was first recognized as a positive site when seven of fourteen students became ill after making a field trip to the area. This site was later to be recognized as the famous rattlesnake hole mentioned so often by the late C. E. SMITH. Unfortunately, the isolation method used to recover the organism from the soil at the site was not described.

SMITH (21) in 1943, and LITTMAN (11), in 1951, developed media more or less selective for pathogenic fungi from clinical materials. There was one drawback to each; both media restricted the growth of all fungi, including the pathogen to be isolated. WHIFFEN et al. (25) in 1946, described an antifungal agent cycloheximide that had been isolated from *Streptomyces griseus*, and PHILLIPS & HENEL (20), in 1950, reported using the antibiotic to control fungus contaminants in bacterial cultures. They also reported that cycloheximide had no effect on the growth of *C. immitis*. GEORG et al. (9) in 1951, formulated a medium for the selective isolation of the organism from clinical materials that contained penicillin, streptomycin and cycloheximide. This medium was used by EGEBERG & ELY (3) and by PLUNKETT & SWATEK (23), in 1956, to isolate *C. immitis* by direct plate methods from the soil of several areas of the San Joaquin Valley.

In 1961, WALCH et al. (24) developed an *in vitro* isolation method for *C. immitis* from the soil, in which he incorporated copper sulfate in the test suspension to further inhibit the saprophytic fungi. This

pretreated soil suspension was plated on a glucose-phytone medium augmented with yeast extract and antibiotics. He used this procedure to demonstrate the presence of *C. immitis* in San Diego, Calif.

From 1954 to 1967 MADDY et al. (12–16) reported on several aspects of the ecology of *C. immitis*. In 1965 MADDY used an *in vitro* plate method and EMMONS' *in vivo* mouse method (modified) for the isolation of *C. immitis* from the soil in Arizona. He concluded that the plate method was not as safe nor as effective as the mouse method.

LEVINE (10), in 1964, sampled the soil from the yard of a residence in Woodville, California, that had been suspected of being the focus of a coccidioidomycosis outbreak among the children of the area. He used four methods of isolating *C. immitis* — two *in vitro* plate methods and two *in vivo* mouse methods. One of the *in vivo* methods was the intraperitoneal technique, the other was a novel method in which the soil suspension was infused into the mouse by the nasal route. He found this the only method of the four to be successful in four of the 37 soil samples tested (approximately 11 %).

In 1965 OMIECZYNSKI et al. (17) reported a method and medium for the isolation of *C. immitis* from sputum. The method was a double pour technique, and the medium used yeast extract as the sole source of nutrient fortified with antibiotics. Yeast extract stimulates the rapid growth and early sporulation of *C. immitis* and has none of the suppressive characteristics of peptone. The medium and method were used to isolate *C. immitis* from soil (18). The method was at least as effective as earlier methods, but the method and medium combined was superior to any of the *in vitro* methods used to that time.

Three artificially infected and seven previously established naturally infected soil samples were used to compare the in vivo mouse technique of EMMONS (7), and three *in vitro* techniques, those of PLUNKETT & SWATEK (23), WALCH et al. (24), and OMIECZYNSKI et al. (18).

MATERIALS AND METHODS

Each of the methods utilizes a 1 : 10 suspension of soil. The EMMONS and WALCH methods prepare the suspension using physiologic saline, while the SWATEK & OMIECZYNSKI methods use distilled water. The methods were compared using aliquots of the same soil suspension prepared in water.

Preparation of the 1:10 soil suspensions

1) Twenty grams of soil was added to 180 ml of sterile distilled water heated to 40° C in a 250 ml screw-capped Erlenmeyer flask.

2) The flask was shaken rapidly for 3—5 minutes.

3) The suspension was immediately poured into a sterile 250 ml graduate and loosely covered with a small beaker. The graduate

was allowed to stand undisturbed for one hour at room temperature (approximately 25° C).

4) A 25 ml aliquot of the topmost supernate was drawn off and transferred to a sterile test tube. All tests were preformed using portions of this aliquot (Fig. 1).

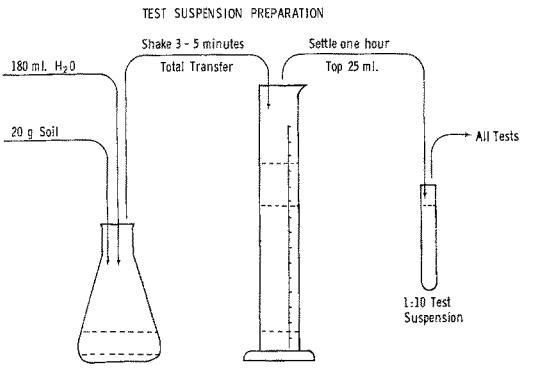

Fig. 1. Preparation of test suspension.

Ten soil samples were tested using the four methods. Three were artificially infected soils and seven were naturally infected soils. The naturally infected soil samples are described in Table I. The

TABLE I

Source and duration of holding time in laboratory of naturally infected soils

Sample No.	Sample source	Time since sample collection
IV	Lakeside, San Diego County	43 Months
V	Lakeside, San Diego County	43 Months
VI	Inyokern, Kern County	76 Months
VII	Inyokern, Kern County	76 Months
VIII	Los Banos, Merced County	54 Months
IX	Los Banos, Merced County	54 Months
X	Shark's Tooth Hill, Kern County	40 Months

artificially infected soil samples were prepared using river-bottom silt obtained near Newhall, California. Three-twenty gram aliquots of this soil were sterilized by autoclaving twice on subsequent days. Spores from one-month-old cultures of *C. immitis,* originally obtained from human cases of coccidioidomycosis were water harvested and one ml of a dilute suspension of each was added to a 20 g aliquot of soil. Viability plate counts were made of each suspension

at the same time. The three soil samples were incubated at 37° C for twelve hours. The soil-spore mixtures were shaken for three minutes. The particulars of the cultures used to infect the artificially inoculated soil samples and the number of viable particles used are shown in Table II. The naturally infected soils used

TABLE II

Viable particles per 20 gr. of artificially prepared soil

Sample No.	Source of patient's infection	viable particles per 20 g. soil
I	Shark's Tooth Hill	1.6×10^4
II	Bakersfield	7.0×10^3
III	San Diego	1.5×10^4

were collected some time ago and have been shown to be positive for *C. immitis* in the past.

Test Procedures Used

a) *The in vivo mouse method of Emmons*:

1) Five ml of each test suspension was drawn up into 5 ml disposable syringes through 20 Ga × 1" needles.

2) One ml aliquots of each suspension were injected intraperitoneally into each of five mice (CF-1) (Fig. 2).

3) The fifty test mice were sacrificed and examined after four weeks.

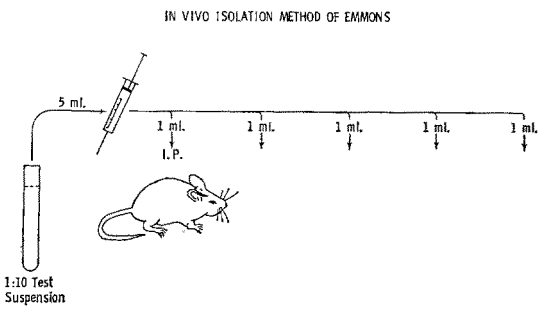

Fig. 2. In vivo isolation method of EMMONS.

b) *The in vitro spray method of Swatek*:

1) Five ml of each test suspension was sprayed onto the surface of five plates of Sabouraud's Agar, using alcohol sterilized "Windex" sprayers.

2) One ml of each test suspension was added to a tube containing 9 ml of sterile water (1 : 100) and mixed by inversion.

3) One ml of each 1 : 100 dilution of a suspension was added to a tube of sterile water (1 : 1000) and mixed by inversion.

4) Five ml of each of the 1 : 100 and 1 : 1000 dilutions of suspensions were sprayed onto five plates of medium in the same manner as were the 1 : 10 aliquots.

5) In an upright position 150 plates were incubated for 24 hours at 37° C to dry and then for two weeks at 30° C (Fig. 3).

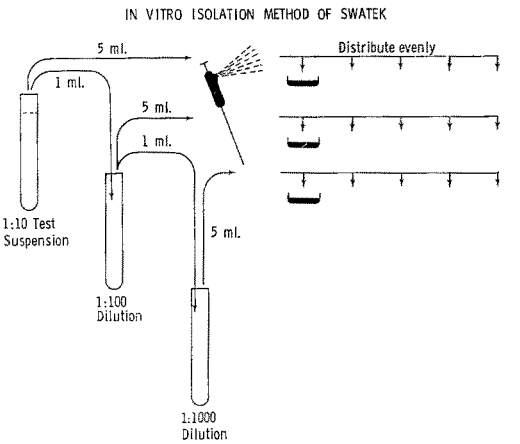

Fig. 3. In vitro isolation method of PLUNKETT and SWATEK.

c) *The in vitro flotation-plate method of Walch*:

1) Five ml of each test suspension were mixed with 5 ml of sterile 0.08 % $CuSO_4$ solution in a test tube (1 : 20). The mixture was incubated at 37° C for 24 hours.

2) The diluted suspensions were mixed by inversion, and one ml aliquots of each were pipetted onto and spread over the surface of the agar of ten plates of Walch's Agar medium.

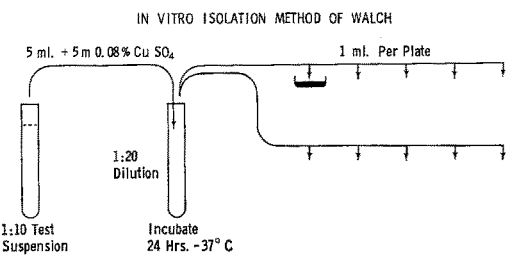

Fig. 4. In vitro isolation method of WALCH.

3) The plates were incubated at 37° C for 24 hours to dry and placed at room temperature for two weeks (Fig. 4).

d) *The in vitro double pour-plate method of Omieczynski*:
1) Five ml of each test suspension was pipetted into ten sterile petri dishes in half ml aliquots.
2) Approximately 15 ml of Yeast Extract Agar was poured into each plate, swirled to mix, and allowed to solidify.
3) A second 15 ml aliquot of medium was poured into each plate.
4) The plates were incubated at 30° C for two weeks (Fig. 5).

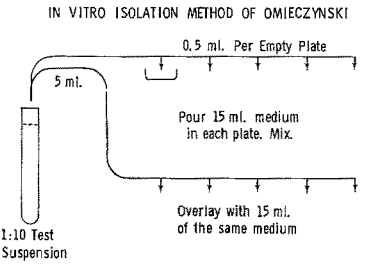

Fig. 5. In vitro isolation method of OMIECZYNSKI.

Medium formulae

The formulae for the media used in each of the three *in vitro* tests are shown in Table III.

TABLE III

Sabouraud's Agar (Modified) (20)	
Peptone (Difco)	1.0 g.
Corn syrup (Karo)	4.0 g.
Agar (Difco)	2.0 g.
Distilled water	100.0 ml.
Walch's Agar (21)	
Yeast extract (Difco)	0.5 g.
Neopeptone (Difco)	1.0 g.
Glucose	1.0 g.
Agar (Difco)	2.0 g.
Distilled water	100.0 ml.
1.5 % Yeast Extract Agar (16)	
Yeast extract (Difco)	1.5 g.
Agar (Difco)	2.0 g.
Distilled water	100.0 ml.

Each medium was fortified with 50 ppm of chloramphenicol (Chloromycetin, Parke Davis Co.) before being autoclaved. After

autoclaving, the medium was cooled in a 50° C water bath, and further fortified with 0.5 mg/ml cycloheximide (Acti-dione, Upjohn) and 0.32 mg/ml streptomycin sulfate (Pfizer). The agar was poured into petri dishes as required for each of the *in vitro* testing methods.

Results

The in vivo method of Emmons

Five mice of the thirty-five injected with suspensions of the seven naturally infected soils were found to be positive for *C. immitis*. All fifteen of the mice injected with suspensions of the three artificially infected soils were positive. The results are summarized in Fig. 6.

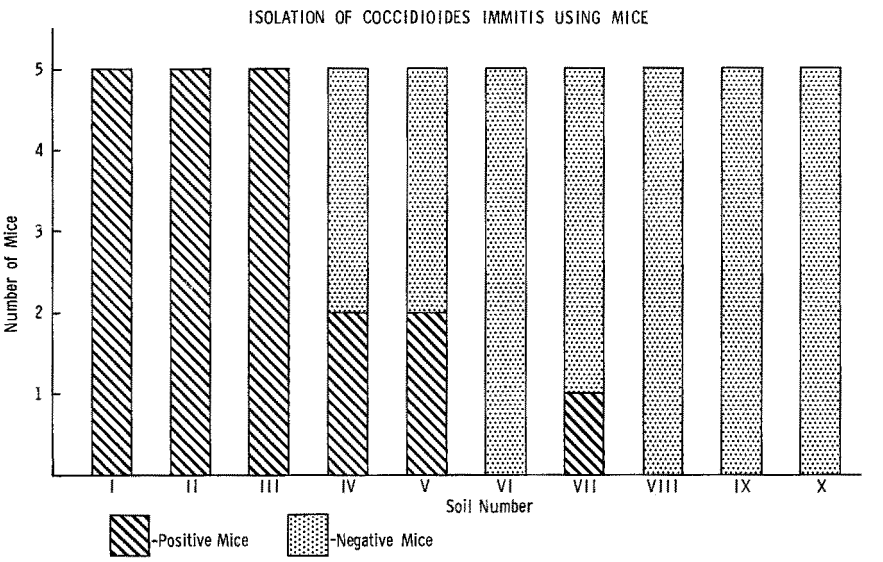

Fig. 6. Results of isolation of *Coccidioides immitis* using mice.

Table IV

Comparison of three in vitro methods for the isolation of *C. immitis*

Method	Total *C. immitis* colonies/*C. immitis* colonies/ml. 1 : 10 Plunkett & Swatek	Walch	Omieczynski
Control Samples (1—3)	400/432.5	162/32.6	408/82
Test Samples (4—7)	12/8.04	3/0.6	27/5.4

The in vitro methods of Plunkett and Swatek, Walch, and Omieczynski

Table IV shows the number of *C. immitis* colonies counted and the calculated number of colonies per ml of 1 : 10 test suspension for each of the three isolation methods. These results are shown in more detail in Fig. 7.

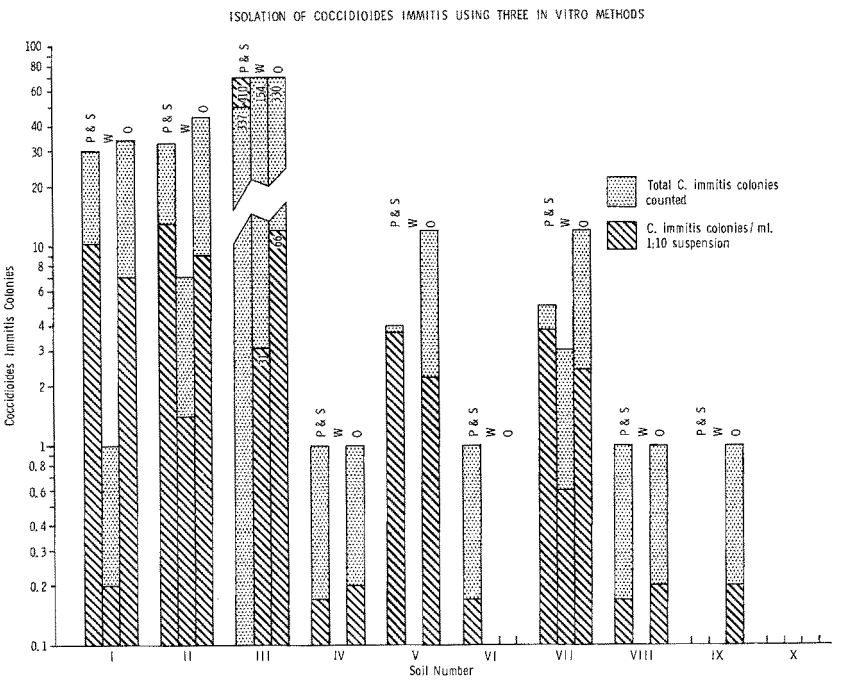

Fig. 7. Results of the isolation of *Coccidioides immitis* using three in vitro methods.

Discussion

The isolation of *C. immitis* from the soil has developed from two basic techniques: (1) direct plating, and (2) the use of animals as differential media. Both techniques may serve special purposes, for example, the animal isolation procedure is an effective method of isolation when cycloheximide is not available. At the same time the isolation is made, Koch's postulates are fulfilled. This technique is not without its limitations, especially in ecological studies where quantitative as well as qualitative estimates are to be made. Also, it follows that the animal serves as a restrictive medium, preventing the isolation of many other fungi, and thus restricting the studies on the relationship of soil population of *C. immitis*. The mouse pro-

cedure serves well as a screening technique for more definitive studies of the ecology where new areas are being investigated. It is also known, both from the literature and from personal experience, that the mouse is not a perfect host for all strains of *C. immitis* recovered from the soil. Sometimes, an organism isolated from the soil and suspected of being *C. immitis* may have to be serially passed in mice several times before *in vivo* endosporulation is observed. The guinea pig, injected intratesticularly with the suspected organism, seems to be more reliable than the mouse for demonstrating the *in vivo* conversion of most strains of *C. immitis*.

The use of direct plating procedures allows the investigator to see the relative numbers of viable particles in a soil sample relative to the other organisms in the soil whose metabolic moiety will allow their growth on the medium employed. The medium used for isolation is selective in many ways, and yet provides the substrate for some organisms metabolically akin to *C. immitis* to be propagated. One cannot carry this concept too far, since experience has shown that a large variety of fungi are capable of growing on most direct plating media. Forms that express metabolic inhibition on the growth of *C. immitis* can also be observed under these conditions. EGEBERG et al. (4), in 1964, reported on several such organisms (*Penicillium janthinellum* and two strains of *Bacillus subtilis*). In direct plating procedures the single most important source of error that results in failure to isolate *C. immitis* is the overgrowth of that organism by soil saprophytes. The double pour procedure has the advantage over other plating methods in that many of the soil saprophytes are trapped deep in the medium. Also with this technique, a large number of soil specimens can be processed at a very low cost per sample.

C. immitis in the laboratory is not a fastidious organism. It grows and sporulates on most bacteriological and mycological media. However, there is a rather wide range of physiological differences among strains of the organism. FRIEDMAN & PAPPAGIANIS (8), in 1956, demonstrated that the sporulation of some strains of *C. immitis* was inhibited by the presence of peptone in the medium. We had earlier demonstrated this inhibition on soil samples from a suspected site in Los Banos, California. On a medium containing yeast extract as the sole source of nutrient, three samples out of twenty-eight tested were found to be positive, whereas no isolations were made on Sabouraud's Agar.

Comparison of results in this paper on the artificially and naturally infected soils using the mouse procedure and three *in vitro* techniques indicates that the mouse procedure is particularly useful in processing highly contaminated soils and in giving unequivocal results rapidly. However, due to strain variation found in commercially available mice and to the low concentrations that occasionally exist in soil specimens, the *in vitro* techniques appear more promising. The necessity of copper sulfate pretreatment used in the

WALCHprocedure seems unwarranted if cycloheximide is available. The results of the comparison study suggest that the use of copper sulfate may be detrimental to the primary isolation of *C. immitis* from soil. The differences between the spray and double pour techniques compared in this paper seem equivocal, although the safety of the double pour procedure would favor its general use. The obvious disadvantage of this procedure in ecological studies, where a comparison of other isolates is necessary, is the abnormal colony appearance in the deep layers of the medium and the lack of sporulation in some instances. The spray procedure carries with it a certain amount of danger from aerosols of spores from the suspension being tested. It is questionable whether the procedure is worth this risk in poorly equipped laboratories. The use of pipettes instead of sprayers reduces the risk, but also reduces the total number of isolates.

Acknowledgements

The authors wish to express their appreciation to Mr. T. DODGE and Mr. R. LOPEZ for the photographic work, and to Miss J. BURNS for preparation of the manuscript.

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