Rapid in Vitro Conversion and Identification of *Coccidioides immitis*¹

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Since many contaminating nonpathogenic fungi resemble *Coccidioides immitis* culturally, isolates from patients must be inoculated into laboratory animals to confirm identification as *C. immitis*. This procedure is time-consuming, expensive, and not generally available in clinical laboratories. When cultures of *C. immitis* were grown in slide cultures on modified Converse liquid medium in purified agar and incubated at 40 C in a candle jar, all 57 isolates demonstrated inhibition of mycelial growth and conversion of arthrospores to endosporeulating spherules in 3 to 5 days. Representative isolates of six species of nonpathogenic fungi that resembled *C. immitis* culturally either did not grow or failed to produce endosporeulating spherules under the same conditions. This procedure is recommended for confirming the identification of a culture as *C. immitis*.

Coccidioidomycosis is a pulmonary disease common in arid regions of the western hemisphere. The prevalence of infection in the population frequently exceeds 50% (5) and approximately one-half of those infected develop symptomatic disease (11). Definitive diagnosis is established by isolating the fungus, *Coccidioides immitis*, or by visualization of characteristic endosporeulating spherules in pathological specimens. Identification of cultures involves the demonstration of arthrospores and reproduction of the disease in laboratory animals. The latter requirement is not academic since many nonpathogenic fungi resemble *C. immitis* in cultural and morphological characteristics (6), and a significant number (15 to 20%) of isolates of *C. immitis* produce atypical cultures (8). Reproduction of the disease in laboratory animals is expensive, inconvenient, and time-consuming (1 to 3 weeks), and, in addition, many laboratories are not equipped to maintain small-animal colonies.

A method more efficient and convenient than animal inoculation would be desirable. Converse (3, 4) described a chemically defined liquid medium in which mycelial phase growth of *C. immitis* would convert reliably to endosporeulating spherules. This required continuous shake-culturing at 40 C with increased CO₂ tension. Roberts et al. (12), using a modified Converse liquid medium and stationary cultures, reported successful conversion to endosporeulating spherules with 201 isolates of *C.*

immitis. This would obviate the need for animal infection, but the method has not been consistently successful in our experience. Brosbe (2) incorporated a modified Converse liquid medium (9) into agar and reported in vitro development of endosporeulating spherules with four isolates of *C. immitis*. This was accomplished in small petri dishes (50 by 12 mm) with incubation at 40 C and in a gas mixture of 20% CO₂-80% air. The Brosbe method should be useful in clinical laboratories. This report concerns confirmation of his findings and modifications of the procedure to make it readily adaptable for use in clinical laboratories.

MATERIALS AND METHODS

Cultures. All 57 isolates of *C. immitis* used in this study were recovered from human cases of coccidioidomycosis. Four of these were primary isolates reisolated from inoculated mice, and the remainder had been transferred in Sabouraud dextrose agar for several generations. Some of these were atypical as described earlier (8). In addition to the proven cultures of *C. immitis*, one isolate of each of the following fungi that resemble *C. immitis* was included: *Geotrichum* sp., *Trichosporon cutaneum*, *Endomycopsis chodati*, *Myxotrichum* sp., *Auxarthron reticulatus*, and *Pseudoarachniotus* sp.

Media. All cultures were grown on glucose-yeast extract medium containing 1% glucose, 0.5% yeast extract, and 1.5% agar. Cultures were incubated at room temperature until arthrospores were present. Converse liquid medium, modified according to the procedure of Levine et al. (9) was used as the suspending liquid for arthrospores and also incorporated into 1% Ionagar no. 2 (MCM-A) for conversion of arthrospores to endosporeulating spherules (2). A

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layer of water agar (1.5%) was used in chambers as a source of moisture for incubating slide cultures. All media were sterilized by autoclaving.

Slide culture procedure. A small block of agar (approximately 10 by 10 by 3 mm) was cut from a poured plate of MCM-A and transferred to a sterile microscope slide in a sterile petri dish. The agar block was inoculated on the upper surface with a loop of arthrospore suspension prepared by adding a drop of Converse liquid medium, as modified by Levine et al. (9), to the top of a culture on glucose-yeast extract medium, allowing the spores to float up, and picking them up with the loop. A sterile glass cover slip was placed on top of the inoculated block of agar and pressed gently to secure it to the agar and to flatten the agar slightly. In separate experiments, the slides were placed either in an anerobic incubator (National Appliance Co., model 3640-5) or in a candle jar. When the anaerobic incubator was used, slide cultures were placed in a moist chamber (a petri dish, 150 by 15 mm) containing a layer of water agar and two applicator sticks to support the slides (maximum of four slides per moist chamber). Dishes were placed in the anaerobic incubator and incubated at either 37 or 40 C with a gas mixture of 20% CO₂-80% air. A small cylindrical jar (18 cm high by 12 cm in diameter) was used as a candle jar. A double layer of filter paper was placed in the bottom and saturated with sterile water to supply moisture. Slide cultures were placed in an upended rectangular glass slide holder (from a staining jar) so that slides remained horizontal. This was placed in the cylindrical jar and a small candle standing in a small glass petri dish was placed on top of the slide holder. A thin roll of modeling clay was attached to the rim of the cylindrical jar. After the candle was lighted, a glass petri dish cover was placed on top of the cylindrical jar and sufficient pressure was applied to seal the unit. The entire unit was placed in an incubator either at 37 or 40 C. Slide cultures were observed at zero time and every 24 h for development of endosporulating spherules. Photomicrography was accomplished with a Nikon Microflex model AFM microscope at $\times 600$ magnification with Kodak Royal Pan film (10.2 by 12.7 cm).

RESULTS

The sequence of morphological changes under different experimental conditions were identical for all of the cultures of *C. immitis* tested. In addition, the sequence of morphological events was essentially the same for all of the nonpathogenic fungi (only minor and nonsignificant differences). Therefore, only typical examples will be described.

***C. immitis*.** Arthrospore suspensions at zero time contained single spores, chains of several spores, and very few hyphal strands (Fig. 1a). When incubated at room temperature, germination and hyphae were apparent at 24 h (Fig. 1b) with branching prominent after 48 h. Most isolates showed typical arthrospore formation in 4 to 5 days (Fig. 1c). When incubated at 37 C

in either the anaerobic incubator or the candle jar, endosporulating spherules were present in 4 to 5 days, but the slide culture was overgrown with hyphae and many newly formed arthrospores had developed. Morphological changes were identical when slide cultures were incubated at 40 C in both the anaerobic incubator and the candle jar. Arthrospores had become round cells at 24 h (Fig. 1d), and these had enlarged and their contents appeared granular at 48 h (Fig. 1e). Endosporulating spherules were obvious in 4 to 5 days (Fig. 1f). Hyphal formation was markedly inhibited at 40 C and no newly formed arthrospores were apparent.

Nonpathogenic fungi. Suspensions at zero time contained arthrospores, singly and in chains, as well as short strands of hyphae (Fig. 2a). Continuous incubation at room temperature resulted in germination, formation of hyphae, and development of new arthrospores in 4 to 5 days (Fig. 2b). A few of these cultures could be easily confused with *C. immitis* by inexperienced personnel. Some species did not grow at 37 or 40 C, and the remainder developed similarly in both the anaerobic incubator and candle jar. Round cells were formed at 24 h (Fig. 2c). These either degenerated in 4 to 5 days (Fig. 2d) or germinated into hyphae with chlamydospores (Fig. 2e) and newly formed arthrospores (Fig. 2f). None ever formed endosporulating spherules.

DISCUSSION

Three primary questions were considered in this study. Would all isolates of *C. immitis* develop endosporulating spherules on the MCM-A medium? Would fungus species that resembled *C. immitis* fail to do this? What were the optimal conditions for consistent morphogenesis to endosporulating spherules and convenient use in clinical laboratories?

All 57 isolates of *C. immitis* converted readily from arthrospores to endosporulating spherules but none of the nonpathogenic species did so. Some of the latter did produce large round cells comparable in size to spherules and, therefore, it is absolutely necessary that an unknown isolate be observed to produce and release endospores (Fig. 1f) before it is identified as *C. immitis*. This was detectable in some isolates by 3 days and in almost all by 4 days. Roberts et al. (12) reported 95% conversion by 3 days with their liquid culture method, so our method is approximately 1 day slower. Significantly, however, we found that the most recent isolates developed endosporulating spherules the most rapidly.

The culture medium and environmental con-

FIG. 1. Morphological stages in the development of *C. immitis*: (a) the arthrospore inoculum; (b) arthrospores germinating after 24 h at room temperature; (c) typical new arthrospore formation after 4 to 5 days at room temperature; (d) round cells formed from arthrospores after 24 h of incubation at 40 C with 20% CO₂; (e) enlarged round cells with cleavage planes after 48 to 72 h of incubation at 40 C with 20% CO₂; (f) endosporulating spherules after 4 to 5 days at 40 C with 20% CO₂.

ditions used in this study resulted in consistent conversion of arthrospores to endosporulating spherules. Although this occurred at both incubation temperatures, there were distinct advantages favoring incubation at 40 C. At this

temperature, spherulation occurred readily and mycelial formation was inhibited (see also references 1, 4). Mycelia developed regularly during incubation at 37 C and, in addition, arthrospores were formed. These represent a definite

hazard for infection of laboratory personnel when slide cultures are removed from the incubation chamber for microscope observation.

This problem was overcome by adding Formalin to the chamber (10 ml for each 1,000-ml volume) and incubating overnight at 37 C. Sub-

FIG. 2. Representative morphological stages in the development of nonpathogenic fungi: (a) the arthrospore inoculum; (b) new crop of arthrospores after 5 days at room temperature; (c) round cells formed from arthrospores after 24 h at 40 C with 20% CO₂; (d) degenerating round cells after 5 days at 40 C with 20% CO₂; (e) formation of hyphae and chlamydospores after 5 days at 40 C with 20% CO₂; (f) germinating round cells, hyphae, and new crop of arthrospores after 5 days at 40 C with 20% CO₂. The fungi illustrated are *A. umbrinum* (a), *A. reticulatus* (b, f), *Pseudoarachniotus* sp. (c, d), and *Myxotrichum* sp. (e).

culturing demonstrated that all cultures were killed by this treatment. This procedure is inconvenient, however, because only a single incubation time can be used or replicate slide cultures must be prepared for each isolate. Therefore, incubation at 40 C is recommended. The increased CO₂ tension has been shown by several investigators to favor development of spherules (1, 10). In this regard, the simple candle jar arrangement proved as effective as the controlled gas mixture.

In our opinion, the slide culture method is more convenient than the liquid culture procedure described by Roberts et al. (12). Liquid cultures required the use of 50-ml Erlenmeyer flasks, preliminary observation through the bottom of the flask on the stage of an inverted microscope to determine that large round cells had formed, and preparation of slides to establish that endospore-forming spherules were present. In contrast, a number of slide cultures can be tested simultaneously in a single candle jar and the slides can be removed daily for microscopy and returned to the candle jar for continued incubation if spherulation has not occurred. Both methods require an incubator set at 40 C, but only a small one is needed for the slide culture method. Although we believe that slide cultures incubated at 40 C do not represent a potential for laboratory infection, some personnel may be uncomfortable with handling such preparations. If so, the original Brosbe method can be used; i.e., MCM-A in small petri dishes and incubation in a candle jar at 40 C. These dishes can be observed daily with low-power objectives without opening the lids and, when large round cells are seen, slides can be made to determine whether endospore-forming spherules are present. This would be an approach similar to that of Roberts et al.: it offers the advantage of smaller culture containers but lacks the convenience of slide cultures.

Finally, one should be aware that the use of refined agar yields better results than the usual bacteriological agar. Brosbe reported that Ionagar no. 2 (Colab Laboratories, Inc.), purified agar (Difco), and agarose (Bausch and Lomb, Inc.) supported conversion of arthrospores to endospore-forming spherules equally well. Our experience has been similar and, in addition, we have evaluated these three agars with respect to formation of endospore-forming spherules. They are rated in descending order of efficiency as follows: Ionagar no. 2, agarose,

purified agar.

The conditions that are necessary to induce morphogenesis of arthrospores to endospore-forming spherules are relatively simple. These consist of the culture medium (MCM-A) and incubation at 40 C in an atmosphere with increased CO₂ tension. The temperature in the incubator around the cultures should be checked. If it exceeds 41 C, little or no growth will result. The agar should be inoculated with a spore suspension sufficiently dense to ensure ease of locating cells undergoing morphogenesis; the denser the suspension, the better the conversion. In our experience, more than 90% of the arthrospores will convert to endospore-forming spherules. Furthermore, since completing these studies, we have taught this method to two persons from other laboratories and they have achieved successful conversions.

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