AN IMPROVED SLIDE CULTURE TECHNIQUE FOR THE STUDY AND IDENTIFICATION OF PATHOGENIC FUNGI

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Received for publication February 2, 1946

The microscopic identification of many pathogenic fungi still depends almost wholly upon morphology, especially upon the size, location, and arrangement of individual spores or groups of spores. The usual procedure is to examine teased wet-mount preparations. These have a number of disadvantages, however. The structures depended upon for the recognition of such fungi as Blastomyces dermatitidis or Sporotrichum schenckii, for example, are often so distorted or disarranged in these teased mounts that identification is made difficult or impossible, particularly for persons unfamiliar with this technique. Slide cultures, on the other hand, when suitably prepared, make possible the definite recognition of these genera, and also help materially with the classification of other varieties of fungi. Use of the available slide culture techniques (Henrici, 1930; Brown, 1942; Lewis and Hopper, 1943), however, is beset with numerous difficulties. None of these methods is suitable for rapid identification of pathogenic fungi.

The present paper describes an improved slide culture technique which is relatively easy to carry out and is useful for practical diagnostic work in mycology, as well as for classroom teaching. Since pathogenic fungi grow slowly, and hence may easily be crowded out by more rapidly developing contaminants, provision has been made to exclude such contaminating organisms. The method also makes possible the direct inoculation of the culture slide with a large inoculum. This has been found to be necessary to insure growth of pathogenic species. Special attention also has been given to the nature of the medium for the microculture. Since identification depends to a large degree upon the presence of typical spores, it is important that the medium used stimulate early and abundant sporulation. Lastly, this improved slide culture technique is designed to permit the easy preparation from the culture of stained slides which preserve intact the morphological features of the fungus. These slides are valuable for making photomicrographs and are especially useful for teaching purposes.

MICROCULTURE TECHNIQUE

Culture media. After some experimentation it has been found that best results in forcing early sporulation of pathogenic fungi are obtained with the specially prepared potato glucose agar described below.

Cut 250 grams of whole potato into pieces about the size of walnuts, place in 500 ml of distilled water, and steam for ½ hour. Pour through a wet towel, make

up to volume, and add to the extract 1.0 per cent glucose and 1.5 per cent agar. No pH adjustment is necessary. Heat until dissolved, tube, and autoclave at 15 pounds for 20 minutes.

Special equipment. The necessary materials for making the microcultures are illustrated in figure 1. The only special piece of equipment necessary is a syringe filled with a paraffin-vaseline mixture for use in making the cover glass supports.

Paraffin and vaseline are melted together in a ratio of 30 to 70 by weight, and the mixture is sterilized in the dry-heat oven. (Such a ratio may have to be varied because of the different grades of paraffin or vaseline available.) This mixture is placed in a sterile 10-ml syringe which has had the tip broken off close to the barrel in order to obtain a larger opening and to permit easier extrusion of the contents.

Preparation of the microculture. The microscope slide is removed from the sterile petri dish setup, figure 1a, with flamed tweezers, and one end is engaged with the slide forceps.

Two parallel strips of the paraffin-vaseline mixture are extruded out across the slide with the modified syringe, figure 1b, to furnish supports for the sterile cover glass. The slide can now be replaced under the petri dish lid and kept there during the rest of its preparation.

A small piece of fungous growth is "raked" off with a heavy nichrome hooked needle and placed on the center of the slide between the paraffin-vaseline lines,
A sterile cover glass is then pressed on the paraffin-vaseline supports with flamed tweezers to form the desired thickness of space underneath, figure 1d. By means of a short-tipped, sterile Pasteur pipette, the melted medium (which has been held at 45 C in a constant temperature water bath) is run under the slide up to or just covering the inoculum so that a good "growing edge" is formed, figure 1e. Finally the lower edge is completely sealed with a layer of hot, sterile paraffin applied with a cotton-tipped applicator, figure 1f.

The petri dish setup now acts as a moist chamber for the completed slide culture when sterile water is added to moisten the filter paper. In order to maintain moisture within the dish over long periods of time it has been found helpful to place inside a pledget of sterile, moistened absorbent cotton.

**Fig. 2. Preparation of Stained Slides**

*Preparation of stained slides.* The slide cultures are examined every day under the microscope until they have reached the particular phase of growth at which it is desired to make permanent preparations. It will be observed by focusing up and down that there is an adherent growth both on the under side of the cover glass and on the top side of the microscopic slide. Since the growth on the cover glass is usually better than that on the slide, an attempt is made to save the former at the expense of the latter in the procedure which follows.

The culture slide is removed from the moist chamber and the paraffin which seals the agar is cut away with a razor blade, figure 2a. The cover glass with its adherent fungous growth is now slowly pushed forward off the microscopic slide by using the end of another slide, figure 2b. The paraffin-vaseline mixture on the cover glass is then cut away with a razor blade, leaving only the agar and fungous growth. This growth that remains attached to the cover glass is fixed by heating in the flame.
The block of agar still on the cover glass may now be removed simply by lifting it off with a razor blade. However, with fungi that produce a heavy mycelial growth, it is better to cut across the base of the aerial mycelium with a razor blade before lifting off the agar. These manipulations leave the heat-fixed aerial mycelium, with all its sporulating organs, relatively intact on the original cover glass without the interfering presence of the agar. If there is adherent paraffin or vaseline, the cover glass may be dipped into xylol. The preparation is now ready for staining and mounting on a new microscope slide with clarite or balsam in the usual manner.

COMMENTS

This slide culture technique has been used for over two years with consistently good results. It has been employed for all the common genera of filamentous pathogenic fungi and also for several varieties of nonpathogenic molds. Certain points are worth noting, however. It should be emphasized again that a large inoculum is essential in making a slide culture of a pathogenic fungus. It is recommended also that three or four microcultures should be set up at the same time in all cases in order to allow for possible breakage, failure of growth, or failure of the fungus to sporulate fully. The method used to guard against contamination of the slide with unwanted fungi is very effective, as the actual occurrence of such contamination is negligible.

Sabouraud’s glucose agar has been used for years by other workers in the preparation of slide cultures, and little effort has been made to secure a medium better suited to the purpose of these cultures, namely, one that will tend to reduce mycelial growth and force early sporulation. Actually, Sabouraud’s medium has an effect exactly opposite to that desired. It stimulates such prolific mycelial growth that sporulation is retarded until very late.

It is well known that special media are required to force sporulation of many true yeasts. Some of these media are potato extract agar; carrot juice agar; carrot, cucumber, beet, or potato wedges; Gorodkowa slants; and vegetable extract agar (Mrak, Phaff, and Douglas, 1942). It may be that some of these media will also serve well in microcultures for the purpose of stimulating early sporulations of fungi imperfecti. But preliminary experiments with several of the aforementioned media indicated that a potato glucose agar is more satisfactory than any of them. By using the potato glucose medium described above sporulation occurred with all the common genera of pathogenic fungi in 3 to 14 days as compared to 10 to 41 days with Sabouraud’s glucose agar. The character of sporulation was entirely characteristic for each genus, and furthermore the spores were not obscured by heavy mycelial growth. (Commercial dehydrated potato glucose agar was found to be unsatisfactory, since it led to the formation of aberrant spore forms or no spores at all.)

The making of good, stained, permanent mounts from slide cultures requires some practice and ingenuity, but the results are well worth the effort (see figure 3). The stained preparations can be used for teaching students the morphology of the different fungi, and in addition they serve ideally for photomicrographs.
FIG. 3. A, Microsporum gypseum (X400); B, Trichophyton rubrum (X900); C, Hormodendrum pedrosi (X900); D, Phialophora verrucosa (X900); E, Coccidioides immitis (X400); F, Blastomyces dermatitidis (X900). All cultures grown from 4 to 14 days on potato glucose agar at room temperature. Fixed preparations were stained with 0.5 per cent safranine.
Good results may likewise be obtained with many of the filamentous non-pathogenic fungi with this slide culture technique. However, the making of good permanent mounts of many nonpathogenic molds is made difficult by the great tendency of these fungi to release the mature spores with the slightest movement.

A few attempts have been made to use this slide culture method for the primary isolation of pathogenic fungi, as it offered the advantages of easy daily observation of the growth with possible early identification, along with freedom from outside contamination. Two cases of ringworm were studied from which the causative fungi were isolated and identified in this manner—Microsporum audouini in 14 days and Microsporum canis in 7 days. It is difficult to say from so limited an experience whether the method is feasible for routine usage in the diagnosis of mycotic infections. It should be pointed out, however, that any medium may be used in these slide cultures, including blood-enriched media.

ACKNOWLEDGMENTS

The author wishes to acknowledge the advice and assistance of Dr. K. L. Burdon in preparing the manuscript. Thanks are also due to Miss Ella May Shackelford for the diagrams and to Mr. C. G. Breckenridge for the photomicrographs.

SUMMARY

An improved slide culture technique is described for the study and identification of pathogenic fungi, in which special provision is made for excluding contaminants. It is relatively easy to prepare.

A new specially devised potato glucose agar is recommended for use with this slide culture for stimulating early and abundant sporulation, in order that the recognition of particular varieties of fungi may be facilitated for the technician and for the student.

A technique is described for making permanent stained mounts from these slide cultures that preserve intact the morphological features of the fungus. These slides are especially useful for teaching purposes and are valuable for making photomicrographs.

REFERENCES