A METHOD FOR PREPARING PERMANENT STAINED MOUNTS OF PATHOGENIC FUNGI FOR MICROSCOPIC EXAMINATION

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Many pathogenic fungi may be recognized grossly by their appearance in culture. The more definitive method of identification, however, is through microscopic examination of the structures on which classification depends: the spores and the sporebearing apparatus. The simplest method of accomplishing this is to tease a small fragment of mycelium out of the culture and crush it under a cover slip. This procedure generally disrupts the spores from their attachments and produces a degree of disorder which obscures the diagnostic relationships of the spores to the structures on which they are borne. Microcultures prepared on slides or cover slips circumvent this difficulty since such preparations can be examined intact. In this way, disarrangement of the fruiting structure is avoided.

The following method is a modification of Riddell (1950) who conceived a means of getting a thin film of fungus to grow over a naked glass surface. The method to be described is simpler and yields permanently stained specimens which are ideal for teaching and research purposes. Although Riddell claimed permanency for his preparations, this has not been our experience with slides stained with cotton blue and ringed with nail lacquer. The quality of the stain gradually deteriorates. Preparations mounted in a lacto-phenol menstruum and ringed with nail lacquer as described by Riddell are only semi-permanent.

METHOD

Thirty ml of glucose-peptone agar (2 per cent glucose and 1 per cent neopeptone) are poured into a sterile petri dish and allowed to harden. The open end of a nonlipped pyrex test tube 12 to 15 mm in diameter is briefly flamed and plunged through the agar at 4 equidistant sites. This results in the excision of 4 cylinders of agar medium. Then each cylinder is dug out carefully with a needle or spatula and placed on the agar surface immediately adjacent to the round hole left by its removal. A suspension of spores or of mycelial fragments is prepared simply by adding a few ml of sterile saline to a culture tube containing the fungus to be studied. The surface of the tube is then rubbed briskly with a needle so that surface spores or hyphal fragments are dislodged into the saline solution. The suspension need not be homogeneous. With a 1 ml pipette, a single drop of the suspension is placed on the surface of each agar cylinder. A 22 mm unsterile, but alcohol clean, square cover slip is briefly flamed and pressed down over each cylinder so that the corners of the slip are equidistant from the center of the disk (figure 1). The petri dish then is incubated at room temperature for a variable period determined by the time required for characteristic structures to develop. It is not desirable to allow the growth to become too abundant. For species such as *Trichophyton mentagrophytes* and *Microsporum gypseum* which grow rapidly and fruit richly, 5 to 7 days are adequate. Two weeks or less is sufficient for most pathogenic fungi.

The virtue of the arrangement is that the mycelium grows radially outward from the periphery of the cylinder onto the underside of the naked cover slip. Therefore, when the slip is lifted from the cylinder at the termination of growth, a ring of mycelium clings to the undersurface of the slip. The hyphae growing over the agar disk itself are imbedded largely in the medium and do not cling to the slip when this is lifted off. The central area of the cover slip thus is empty in the area corresponding to the disk. Peripheral to this naked circle is a radial fringe of mycelium which is usually thin enough to allow uniform mounting on a glass slide.

The staining and mounting of the cover slip is accomplished in the following way which represents a modification of a method previously described for the differential staining of fungi (Kligman and Mescon, 1950). If a large number of cover slips are to be processed at one time,
special staining dishes are available which hold about 10 cover slips at one time.

1. Immerse for one minute in 95 per cent alcohol.
2. Immerse for 5 minutes in 5 per cent aqueous periodic acid.
3. Rinse briefly in tap water.
4. Immerse for 2 minutes in the following solution: basic fuchsin, 0.1 g; alcohol 95 per cent, 5 ml; water, 95 ml.
5. Rinse briefly in tap water.
6. Immerse for 1 to 2 minutes in the following solution: zinc hydrosulfite, 1 g; tartaric acid, 0.5 g; water, 100 ml.
   (Zinc hydrosulfite may be obtained from the Virginia Smelting Company, W. Norfolk, Virginia. This solution generates sulfur dioxide and may be used if kept in a closed bottle for as long as there is a strong smell of sulfur dioxide.)
7. Immerse for 1 minute each in two changes of tap water.
8. Immerse in 95 per cent alcohol for 10 seconds, 100 per cent alcohol for 1 minute, and 2 changes of xylol, each for 1 minute.
9. Mount in clarite or Harleco synthetic resin mounting medium.

The fungi are stained red. Some representative examples of the results achieved with this method are shown in figures 2 to 4.

**Figure 1.** The agar cylinders have been cut out and deposited on the surface of the medium. Three of the cylinders have been inoculated with a spore suspension and covered with a cover slip.

**Figure 2.** Macroconidia of *Epidermophyton floccosum*

**Figure 3.** Macroconidia of *Microsporum gypseum*

**Figure 4.** Tuberculate conidia of *Histoplasma capsulatum*

**DISCUSSION**

The spores of some species of dermatophytes are produced poorly or not at all on routine media such as the one given above. Certain growth substances and vitamins may enhance their production (Georg, 1949). Brain-heart infusion-tryptose agar (blood agar base, Difco) to which have been added 1 per cent glucose and 5 per cent human or horse blood has encouraged spore formation in some instances when the glucose-peptone medium failed to give satisfactory results. This has been particularly true of *Phialophora verrucosa*, *Trichophyton rubrum*,
Trichophyton faviforme, and Trichophyton violaceum. Freshly isolated cultures of all species yield the best results.

SUMMARY

A method is described for growing fungi on the surface of cover slips and staining them with basic fuchsin so as to result in the preparation of a permanent slide.

REFERENCES