OBSERVATIONS ON MYCOLOGIC TECHNIQUE WITH PARTICULAR REFERENCE TO PATHOGENIC FUNGI *

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One of the most formidable obstacles to recognition of the taxonomic position of pathogenic fungi is the lack of a standard technique which will bring out their distinguishing characteristics. The object of this paper is to assist the beginner in recognizing current organisms encountered in medical mycology.

A METHOD OF ISOLATING PURE CULTURES FROM SKIN LESIONS

Our practice, after cleansing the skin with ninety-five per cent alcohol followed by ether, is to scrape the lesion with a sharp full-bellied, sterile scalpel, or to shave it with a sterile safety razor to the point where bloody serum just begins to ooze. It is best to hold a sterile Petri dish below so that the scrapings may fall within, or if none fall, to remove with another blade such scrapings as may have adhered to the knife or razor and drop them in the dish. The dish is now covered and delivered to the laboratory with name, date, and clinical data. Photographs or drawings of the lesion make the case far more complete.

Upon reaching the laboratory, and after having removed fragments for maceration in potassium hydrate solution (forty per cent) for direct examination, just enough Sabouraud maltose or glucose agar is poured into the plate containing the scales and detritus as will form a very thin film on the bottom. The scales as they lie in the plate are thus caught by the nutrient medium. To reduce chances of bacterial contamination the scales may be covered with alcohol for a short time previous to sowing, but this does not reduce the chances of contamination by hardy saprophytic fungi which may have lodged on the skin, and may prevent the growth of the causative pathogen.

Upon the development of colonies just visible to the naked eye, those that are suspicious are circled with a glass writing pencil, each being given a serial number. The marked colonies are then

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transferred to Sabouraud's proof media slanted in tubes, as well as to our conservative medium which is sugar-free. The transfer is made to prevent the overgrowing of promising young colonies by rapidly growing contaminating organisms such as Aspergillus and Penicillium.

To obtain pure cultures from these transfers on agar slants, the colony, if soft, may be touched with a sterile platinum needle or loop, emulsified in a few cubic centimeters of any liquid nutrient medium, and from this tube Sabouraud agar tubes can be inoculated, cultures being poured into Petri dishes by the three-plate Koch method. In practice, however, we have found but two plates necessary.

If, on the other hand, we have to deal with a hard, tough, pasteboard-like colony, a bit should be worked loose by a sterile platino-iridium hypodermic needle set in a glass rod, and mashed, cut, and disintegrated against the inner wall of a tube containing a liquid nutrient medium, incorporating the pieces and shreds with the fluid. This transferred material is left to macerate a day or so, with occasional shaking to disarticulate some of the mycelial elements. On recognition of sufficient growth in this medium it is shaken and Sabouraud plates are prepared as in the case of soft colonies.

When a valuable organism persists in living in intimate symbiosis with a banal one, such as a plant saprophyte or a coccus, there are still ways by which the contaminating organism may be eliminated. First, and perhaps the most effective, is to raise the acidity of the medium. By increasing the acidity of a medium, bacteria and some fungi may cease to flourish while the pathogen may thrive. Second, baking at 50° C may be resorted to. Finally, chemicals and dyes may offer a solution.

On development of cultures in these plates, suspicious colonies are fished and transferred to Sabouraud proof agar slants and in the vast majority of cases they will be found to be pure cultures.

A METHOD OF ISOLATING FUNGI FROM FECES, TONGUE-SCRAPINGS, ETC.

This is practically Anderson's technique. Poured plates are allowed to harden first and then twenty-five points of contact are made in each by a platinum loop repeatedly soiled in the material. After about four days, when colonies have developed, these are fished and all apparently different species purified by the Koch method. In this way the percentage of points of contact of the material with

the medium which contain similar colonies gives a rough idea of the abundance of colonization in the material.

HANGING BLOCK CULTURES

It is from the preceding pure colony plates that the hanging block may be made, preferred by us to the hanging drop, which, in closely adherent colonies, with deep-rooted mycelium has manifest disadvantages. In making the hanging block two young colonies should be cut out of the agar with the platino-iridium needle and each set upon a sterile cover-slip, enough 0.2 per cent glucose broth being previously placed in the center to keep the block moist. These young colonies should be removed without too much alteration of their contour. Two micro slide glass rings, 15 mm. in diameter and 3 mm. in height, are now mounted on a glass slide, two by three inches. The rims of these rings are painted with sterile vaseline, the lower rim to seal the ring to the slide, the upper rim to the inverted cover slip bearing the hanging block, thus producing a sealed chamber for the growth of the organism.

Although the hanging block is considered to be one of the most precise methods for classifying these organisms, it has the disadvantage of being confined in a sealed chamber containing only a definite quantity of oxygen and when this is consumed an aerobe is often repressed in its growth. Nevertheless, most beautiful mounts can be secured, and indefinitely preserved, by exposing the hanging culture first to cosmic acid vapor then to alcohol fumes, mounting in Farrant's medium, and asphalting the inverted cover slip over the concavity of a hanging drop slide (Weideman's method).

PLATE CULTURES

The examination of young plate colonies has come to take a more and more important place in the classification of fungi in our laboratory. One of the chief advantages it possesses over the hanging block is that we can utilize a step in the necessary purification of our original culture without the additional laborious and delicate work of preparing the hanging block. Moreover, in the plate there is more spreading of the colony and the fertile hyphae are more readily found and clearly identified. In the plate there is a fairly efficient aeration through minute imperfections in the fitting of rim and cover. This prevents stunting of the colonies. There is, of course, more chance of contamination from air-borne spores, but if

carefully handled and kept out of a breeze, these plates remain uncontaminated for a long time. There is the objection that on removing the cover to examine with the higher power of the microscope, the plate is almost sure to be contaminated, thus precluding a reexamination, but the plate colonies may be first viewed from the under surface with a 16 mm. objective and only opened when this rough preliminary inspection denotes unmistakable fructification or a characteristic arrangement of elements. Then at one sitting the plate may be opened and the colonies examined, described and sketched under higher power objectives.

The hanging block is preeminently useful for following the development of the organism. The plate seems to us much more practical for rapid classification. With the 16 mm. objective it is rarely possible to be sure of the genus. Only by removing the cover and using the 8 mm. lens do necessary details become apparent. By exposing for a time to formol vapor and covering a colony with a drop of cedar oil, it is possible to examine with the oil immersion and take accurate measurements in addition to securing greater richness of detail. Indeed, a cover-slip may be interposed and the colony often successfully examined in the usual way by oil immersion lens.

The size, shape, surface topography, and consistency of a plate colony are not always dependable for the determination of the taxonomy. If a pure colony is not well diluted originally in the poured plates, the colonies are apt to be small, close together, and lacking in their distinguishing characteristics. A mycelium-forming fungus often produces perfectly round colonies consisting alone of yeast-like forms in the same plate with others that are heavily fringed with branching hyphae. The latter are especially noted in colonies in duress (*"les cultures en souffrance"* of Brumpt) which are often deeply imbedded in the agar and present as ovals, crosses, triangles, etc., with fringe. In the same plate some colonies may be dull and lustreless, while the remainder are shiny. It is very common to find a high percentage of colonies which are powdery and a few which are smooth, shiny, and puckered. It is not rare to find certain colonies which spread in concentric rings while others do not so spread. On two occasions the writer has made subcultures in Sabouraud slants from fifteen colonies, different the one from the other as above described, and after development the slants became macroscopically identical.

From plates in which contents have been well diluted originally, and in which colonies are few and far between, the abundance of

food and lack of inhibition the one upon the other have permitted characteristic development.

Another consideration of importance in using plate cultures to bring out normal morphology in growth is the ease with which drawings can be made. The sketches made from a drop of a bouillon culture mounted between cover slip and slide utterly fails to bring out normal growth relations between the constituent elements. Photography is more difficult owing to the impossibility of focussing any considerable number of elements in the same plane.

A COMPARISON OF SABOURAUD'S PROOF AND CONSERVATIVE MEDIA

Sabouraud's proof medium contains four per cent of glucose or maltose in a nutrient agar. The addition of certain sugars, especially glucose and maltose, to agar media not only stimulates the vegetative forms but brings out color, as well as producing in general a more luxuriant growth. This is a valuable aid to early differentiation of common non-pathogenic fungi, such as Aspergillus, Penicillium, Sterigmatocystis, etc., from others suspected of being the causal agent of a particular disease.

In investigating dermatomycoses the scales are often sown in conservative media to prevent the formation of a duvet and the development of pleomorphism, as once a fungus becomes pleomorphic the true characteristics are permanently lost and do not return either on subcultures nor after passage through animals. When pleomorphic, certain genera, such as Trichophyton, Microsporum, and Epidermophyton, blend into a colorless picture of tangled mycelium with scanty spore formation and their generic marks are obscured. It may be said, however, that many duvet-producing genera often clearly reveal all of their reproductive peculiarities on the first sugar agar medium; it is only in time and after repeated subculture on sugar media that these organisms become pleomorphic.

It is rarely possible to classify an organism even approximately, solely on the appearance of its sterile mycelium. Fertile hyphae are necessary. Therefore, in all fungi which tend to run to pleomorphism and duvet, stock cultures should be preserved on conservative media.

The conservative medium, which from its lack of sugar stimulates the production of resistant forms, especially of asci, has another application in the separation of Saccharomyces from Torula and Cryptococcus. One of the best means of eliciting ascus-formation in Saccharomyces is the use of Gorodkowa's medium which consists in

adding 0.2 per cent glucose or maltose to a special nutrient agar for the purpose of starting the colony, the sugar content being soon consumed and the organism being forced by starvation into spore formation. Until a yeast has been proven not to form asci the doubt as to its classification among the Saccharomyces remains, and these media compel ascus formation in the latter genus.

It is significant that intestinal fungi do not prosper at all well in conservative media as they seem to require an abnormal amount of sugar.

MACROSCOPIC CHARACTERISTICS OF CULTURES OF FUNGI

The great desideratum is to secure a clear medium for the observation of mycelial downgrowth and to facilitate observation of small plate colonies. The macroscopic appearance of a mycologic culture has a tremendous importance in classification, far more so than in bacteriology, provided that the culture media is universally standardized and that the media employed in an investigation is specified. Important characteristics are exuberance of growth, color, fluorescence, translucence; surface topography, whether raised or flat, granular, smooth, shiny, dull, veil-like, furrowed, radiate, verminous, honey-combed, pitted, velvety; whether moist or dry; as to contour, whether regular or irregular, circular, fringed, or sharply delineated, etc. The observation of these macroscopic characteristics is begun in the plate and completed in the sugar slant and giant culture.

PREPARATION OF SABOURAUD'S MEDIA

It is desired to give our technique exactly as practiced in our laboratory rather than suppress details which we believe after long usage are responsible for a sterile, clear agar.

It is not economical to make up less than five or six liters at a time, yielding, roughly, about 600 tubes.

Place in each of three two-liter Ehrlenmeyer flasks 40 grams of agar, 20 of peptone, and 1,500 cc. of water. Now place these in an Arnold sterilizer for thirty minutes or until the agar is dissolved. At this time, if desirable, the pH may be adjusted before cooling. We do not make this adjustment for the simple reason that our method brings regularly a pH of 6.5 to 6.6. In cases where the pH needs to be accurately known before sowing, a tube is taken from the batch and its pH is determined.

When the media has cooled to 50°C., 75 grams of egg albumin,

shaken in 1,000 cc. of water is added and stirred in. The three flasks are now placed in the Arnold sterilizer for an hour at 100°C.

The coagulum formed will clear the medium which is now filtered through cotton, or cotton and cheese-cloth. At this point the quantity of filtrate is measured (there will always be a loss by evaporation) and the quantity of sugar sufficient to make a four per cent solution is dissolved in the missing quantity of water, and added to the hot filtrate.

The medium is now distributed in tubes and sterilized in an Arnold sterilizer at 100°C. for forty-five minutes on each of three successive days. The tubes can now be slanted on a cooling rack.

GIANT CULTURES

The advantages of giant cultures, of course, lie entirely in their macroscopic peculiarities which are brought out with a vividness not possible to secure in the plate and slant. We formerly employed for this purpose the Ehrlenmeyer flask but we have abandoned this in favor of the pyrex glass Kolle culture flask used in the manufacture of typhoid-paratyphoid vaccine at the Army Medical School. These cultures last a year or so, can be easily handled without danger of contamination or breaking, are economical of space, since they can be stored one above the other, and are most useful for teaching and for photography.

GELATIN CULTURES

In the stab culture, which we make routinely, the Torula and Cryptococcus give a straight line without lateral extension, termed in our laboratory, a nail culture.

The Saccharomyces give a nail culture with lateral nodosities or a fine, almost imperceptible, lichen-like extension.

Monilia psilosis and Monilia krusei grow abundantly and produce long, fine, needle-like extensions from the nail axis which become shorter toward the tip of the stab, the "inverted pine tree".

Monilia parapsilosis is similar to the above with the difference that the mycelial extensions give off lateral branches and sub-branches in abundance, yielding a much denser ramification, the "inverted fir tree".

The intestinal Mycodermata (we have adopted Brumpt's suggestion of excluding the term "Oidium" from medical mycology) usually liquify gelatin after a preliminary pine or fir tree, some slowly, others very rapidly.

PREPARATION OF GELATIN MEDIA

To 900 cc. of nutrient broth add 200 grams of gelatin and heat in a water bath until dissolved. The Bacto-gelatin and Pfanstiehl's brand are highly acid, about plus 3 or 4 to phenolphthalein. Adjust reaction to about 6.5 or any desirable pH. Let cool to 50°C., add the whites of two eggs shaken in 100 cc. of nutrient broth and bring it rapidly to a boil at 100°C. for ten minutes in a double boiler with saturated salt solution in the lower compartment. The cake formed should clear the solution. Filter through cotton, or cotton and gauze if necessary. Distribute in tubes and sterilize at 100°C. for fifteen minutes on each of three successive days. Cool rapidly after withdrawal from the sterilizer to obtain a hard gelatin, not liquified at our room temperature, or in our seeding room where temperature is generally 28°C.

FERMENTATION OF SUGARS

The incorporation of from one to four per cent of sugars to peptone water yields valuable infomation of a biochemical nature. In the past we have used nutrient bouillon as a basis for carrying these added sugars but for simplicity's sake it is preferable to use peptone water. The important feature of testing fermentability is to set the pH at about 7., and note the changes in reaction at regular intervals under the influence of the culture. In a long line of experiments conducted in 1914–16, Ashford described the formation of gas and rise in acidity of certain of these media due to culture therein of Monilia psilosis, and its subsequent fall to neutrality throughout a period of about two weeks. Only six sugars were thus affected; glucose, levulose, maltose, galactose, saccharose and dextrin, the first three regularly, the last three very irregularly. All other sugars failed to ferment. On extending these investigations it was found that of all the common intestinal fungi only Monilia psilosis and certain species of saccharomyces fermented maltose. On this account maltose was chosen as the standard in the study of monilia psilosis.

In investigating other fungi, however, all six sugars in addition to inulin, raffinose and mannite should be used. Arabinose, dulcite, iso-dulcite, and the rarer and extremely expensive higher sugars are infrequently fermented and need not be currently employed. There are very few lactose-fermenting fungi but the brilliancy and morphologic variety of fungi in this and the higher sugar media makes culture therein well worth while. The easily fermented sugars

bring out the yeast-budding fungi and the various resistant forms are held in abeyance.

But the disadvantage of depending upon fermentation tests to distinguish species, especially of Monilia, is that such tests are not constant; species which have once or twice fermented certain sugars with gas production have often failed to so act on a subsequent occasion, and vice-versa. We have proven that ultraviolet rays will destroy fermenting powers in Monilia psilosis and the sunlight will act similarly.

In addition to these evidences of fermentation, gas production and increased acidity of the medium, there are certain other important phenomena in bouillon culture worth noting. The nature of the deposit is at times a clue to grouping of fungi; this may be granular and sand-like, or grumous, or sputum-like, or thready. Fungi rarely cause turbidity of the medium. Then there are genera and species which form a pellicle with characteristic peculiarities, or a series of pellicles which fall in succession toward the bottom of the tube, or which grow upward in duvet form to fill the tube above the level of the fluid, or which form a collar in contact with the glass at the surface, or which form no pellicle at call. Care must be taken to leave a culture at least two weeks before reporting negatively as to gas production. Dunham's U-tubes have proved most useful in our hands and may be set in slots cut in blocks of wood holding from six to eight tubes.

LITMUS MILK

For some time we used this medium for intestinal fungi but the sameness of results, i.e, eventual alkalinity, generally with a very brief and doubtful preliminary increase in acidity without coagulation in the case of Monilia psilosis, caused us to abandon its use as a routine procedure. But it should be always employed in trying out a new or doubtful species. The cow's milk, which we generally receive in an acid state, is slightly alkalinized with N/10 sodium hydroxide, partially freed of its fat, sedimented, and sterilized after adding sufficient azolitmin as an indicator. Important points to note are changes of color, coagulation, and peptonizing of the clot.

VITAL STAINING

This method, while not new, is not as commonly employed in studying bouillon cultures under the oil immersion lens as it should be. Its manifest value lies in sharpening structural characteristics

within the thallus and spore, and in bringing out selectively the chemical composition of internal organs while the organism still lives.

While we do not fail to recognize a certain value in the study of fixed and stained fungi, in practice we consider the study of the unstained or vitally stained organism of far more value, and our conclusions as to morphology are largely, indeed almost exclusively, thus determined. The examination of a drop of the bouillon culture is also a check upon the purity of the growth.

The preparation of slides for vital staining is of paramount importance. They should be new, free from blemishes, and should be freed of fat by the *sulphuric-acid-bichromate* method. After neutralizing, they should be cleaned with a fat-free cloth. Previous to use, remove all dust with a new camel's hair brush washed with ether and dried. Place one or two drops of Janus green, neutral red, or Scharlach R solutions of 1:2500 in ethyl alcohol, and let them extend over the entire slide, which they will do if the latter is fat-free. Now dry in the air. A drop of the liquid media containing the fungus is placed upon a cover slip and inverted upon the slide containing the stain. Let settle and then rim the edges with vaseline. Examine at intervals.

STAINING OF FIXED SPECIMENS

The staining is intensified by substituting a drop of clear blood serum for the liquid medium and emulsifying in it material obtained from the culture on solid media. The current blood stains may also be used (Wright's Giemsa, Leishman) as follows:

With the platinum loop mix enough of the colony from a Sabouraud slant with a drop of clear blood serum, placed at the end of the slide. Before it dries spread it gently with another slide, thus producing a thin film. Fix with methyl alcohol for from one to three minutes. Stain with the above mentioned blood stains and use for washing or diluting the stain neutral water. The stain should remain for from five to fifteen minutes. Differentiate with acetic acid solution, $1 \times 1{,}000$ a few seconds only. Wash well. The addition of serum prevents undue shrinking of the cell.