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LONG TERM OBSERVATIONS ON PATHOGENIC FUNGI CULTIVATED ON ARTIFICIAL MEDIA FOR TWO, THREE, FOUR AND FIVE DECADES. PERMANCY AND VARIATIONS OF THEIR CHARACTERS. THE "STERILE DISTILLED WATER METHOD" OF CULTIVATION TO MAINTAIN SUCH FUNGI IN MYCOLOGICAL COLLECTIONS AND PREVENT THE DEVELOPMENT OF PLEOMORPHISM

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LONG TERM OBSERVATIONS ON PATHOGENIC FUNGI CULTIVATED ON ARTIFICIAL MEDIA FOR TWO, THREE, FOUR AND FIVE DECADES. PERMANCY AND VARIATIONS OF THEIR CHARACTERS. THE « STERILE DISTILLED WATER METHOD » OF CULTIVATION TO MAINTAIN SUCH FUNGI IN MYCOLOGICAL COLLECTIONS AND PREVENT THE DEVELOPMENT OF PLEOMORPHISM (*)

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SVMMARIVM — Pathogenos mucores si artis auxilio aliquot per annos tractemus tres familias obtineamus: 1) familiam cuius characteres semper iidem sunt; 2) familiam cuius characteres cum mutentur tum reversibiles esse ostenduntur; 3) familiam cuius characteres funditus mutati pleomorphismus Sabouraudi gignunt.

Illud praeterea est notatum quod mucores plerumque pathogeni, in illis omnes dermatophyti, si in stillata aqua, quae sterilis sit, serventur, in annum vitalem spiritum non amittunt, cum in stillata aqua pleomorphismum non gignant.

INTRODUCTION

At the beginning of this century and up to the early twenties, the majority of mycologists and bacteriologists believed in the « fixity » of mycetes and bacteria: the cultural

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and physiological characters of these organisms would not vary significantly if grown under standard nutritional and environmental conditions. The only change admitted, a biological one, was a loss of pathogenicity in old strains. Then the pendulum swung the other way and a flow of publications commenced, which, continuing unabatted to the recent day, has detailed the spontaneous changes and variations of fungi cultivated on artificial media.

The purpose of this communication is to make a few remarks on the subject, based chiefly on the study of certain pathogenic fungi isolated long ago and kept under practically continuous investigation ever since, being subcultured on the average once a month on dextrose agar (four per cent until 1930, then two per cent). The intention is merely to quote facts and to relate observations without making any attempt at an explanation of their causation.

In my experience pathogenic fungi of human origin can be separated into three groups:

1) Those whose characters do not change or only in a minimal way, through the years of subculturing on artificial media.
2) Those whose characters may change, even profoundly, but which after intervals, usually long, resume their original characteristics.
3) Those whose characters change permanently and irreversibly.

GROUP I. — *Fungi with Permanent Characters.*

The expression « permanent characters » must be taken *cum granu salis*. Is there anything in nature which is absolutely changeless and immobile? Doubtless changes occur all along, but they are minimal, and for practical purposes may

be ignored. As examples of this group of fungi mention may be made of certain species of *Candida,* particularly certain strains of certain species, e.g. *Candida krusei* CASTELLANI (1910), BERKHOUT, 1923. The original strain isolated from sputum in Ceylon in 1909 is still extant. When first isolated, it produced on glucose agar a growth which was rather dry with a finely granular or very delicately creased surface.

Microscopically the free yeast-like cells were ovoid or somewhat elongated and pseudomycelium was formed. Among the six « sugars » which are at present generally employed for yeast identification — namely, dextrose, maltose, galactose, saccharose, lactose and inulin — it produced fermentation with gas only in dextrose. Among other sugars apart from the standard six it fermented only fructose and mannose. This strain has been re-investigated by a number of workers, among whom are SPAAR (1926), ZEPPONI (1931), URSO (1950), and CAPOCACCIA (1956). No change has been noted. At present fifty years after isolation, the strain produces a colony on dextrose agar which is somewhat dry, and its surface is finely granular or very delicately crinkled. Microscopically the cells are ovoid or elongated: of the six standard sugars only dextrose is fermented, and of the other sugars only fructose and mannose.

Still extant also, in the author's collection, are the original strains of *Candida tropicalis* (CASTELLANI 1910) BERKHOUT 1923, *C. pseudotropicalis* (CASTELLANI 1911) BARGAL 1931, *C. macedoniensis* (CASTELLANI 1919) BERKHOUT 1923. Like *C. Krusei* they have remained unchanged culturally and biochemically through the years of cultivation on dextrose agar. An intersting point is that *C. tropicalis,* which was found definitely virulent and pathogenic for laboratory animals on first isolation, is still so after five decades of continuous subculturing (URSO, 1961).

It would appear also that antigenically the original strains of *Candidae* isolated so long ago have undergone very little, if any, change. In 1934, at the Ross Institute in London,

Dr. Mackenzie DOUGLAS and the author carried out a serological investigation of *Candida* fungi by means of agglutination and complement fixation tests. They recognised four serological groups:

Serological Group I comprising *C. albicans* and *C. tropicalis*;

Serological Group II comprising *C. macedoniensis* and its varieties;

Serological Group III comprising *C. pseudotropicalis* and its varieties;

Serological Group IV comprising *C. krusei* and its varieties.

Groups I, II and IV were well-refined and clear-cut; Group III far less so, in fact badly defined.

A few years ago, in my laboratory, repeated investigations gave practically the same conclusions. Recently, intensive serological studies on the yeasts in general, including *Candidae,* have been carried out in a number of scientific institutes in America, England, Japan and other countries, and in Germany an excellent monograph on serological mycology has been published by Hans SEELIGER.

GROUP 2. — *Fungi Showing Reversible Changes.*

In this group there may be mentioned as examples *Trichophyton balcaneum* CASTELLANI 1916, and *Geotrichum matalense* CASTELLANI 1915, the original strain of both of which still exist.

Trichophyton balcaneum was isolated during the First World War, in the Balkans from cases of a peculiar, diffuse, scaly condition of the scalp, resembling a severe form of pityriasis sicca rather than tinea. It grew on dextrose agar, producing a somewhat nodular or crinkled or slightly convoluted

flattened, glabrous, dirty-whitish or beige colony; it liquefied gelatine rapidly; it clotted milk. Microscopically, no macroconidia, no spirals and no nodular or denticulated bodies were seen; only mycelial threads with a few very doubtful microconidia. The fungus continued to show the cultural characters mentioned above for a number of years until 1928, when definite changes appeared; the colonies became covered with whitish duvet (aerial mycelium), not very long but quite abundant, and on scraping it off, the growth appeared as a smooth, flattish mass, not nodular nor crinkly, not convoluted in parts. It appeared to have become pleomorphic. These new features remained inchanged for about a year, and then the aerial mycelium disappeared and the colonies began to show again a glabrous, somewhat knobbly, slightly convoluted aspect. A few years later, the colonies once more became fluffy, and since then these totally different appearances of the colonies have alternated at long irregular intervals of years, although the medium has always been the same, i.e. dextrose agar four per cent until 1930, later two per cent. At present the cultures have the same appearance as on first isolation. The microscopical characters have never varied, macro-conidia, spirals, nodular and denticulated bodies and other specialised structures have always been absent.

Geotrichum matalense CASTELLANI 1915. The original strain isolated in 1914 is still in the author's possession. When first isolated, and for years after, the cultures on dextrose agar appeared fluffy and whitish, and when this duvet was scraped off the surface of the colony was smooth. Then in 1928, after continuous subculturing on dextrose agar for 13 years (approximately once a month), the duvet disappeared and the growth became deeply rugose and somewhat convoluted in parts, with a smooth, glabrous somewhat moist-looking surface.

Some years later, in 1934, the original characters with plenty of duvet came back. In 1936, the duvet again disappeared, to return a few months later. At present the cultures show absolutely the same characters as when first isolated, with abundant whitish aerial mycelium present.

GROUP 3. — *Fungi Showing Irreversible Changes.*

Every medical mycologist is well acquainted with the irreversible changes shown by certain dermatophytes when grown for long periods on artificial media, especially dextrose agar. The fungus loses its original characteristics, becomes fluffy, and microscopically macroconidia, microconidia, spirals, modular and denticulated bodies are no longer seen, only sterile mycelium being present. The original characters never return; in fact, it may be said that new degenerate races, possibly species arise with features of their own which remain permanent. The phenomenon was first studied by SABOURAUD who introduced the term « pleomorphism », not to be confused with « polymorphism », to indicate it. Once a fungus has become pleomorphic, it remains so indefinitely. The subject of pleomorphism has been treated thoroughly in many books, monographs, and innumerable scientific papers, especially by French authors, and recently in America by REISS and LEONARD (1956). Long ago, SABOURAUD (1910) proclaimed that the use of a poor medium is the best way of preventing pleomorphism, and for this purpose he devised his « maintenance agar », which is prepared with peptone water instead of broth and contains no dextrose or any other sugar. None of the media recommended subsequently, including the so-called natural media and media containing various chemicals and antibiotics, have been completely successful-far from it.

In recent years, a very simple procedure for the prevention of pleomorphism has been introduced by me, which so far

seems to be successful « cultivation », so to speak, in plain sterile distilled water. It is based on certain experiments carried out in the Mycological Department of the London School of Hygiene and Tropical Medicine in the years 1938-39, the results of which were published with the title *The Viability of Some Pathogenic Fungi in Sterile Distilled Water* (CASTELLANI, 1939).

EXPERIMENTAL

On July 5th 1938, 12 tubes of sterile distilled water were inoculated severally with the following fungi: *Candida krusei* Cast., *C. albicans* Robin var. *pinoyi*, *C. tropicalis* Cast., *C. pseudotropicalis* Cast., *C. macedoniensis* Cast., *Geotrichum rotundatum* Cast., *G. matalense* Cast., *G. asteroides* Cast., *G. rugosum* Cast., *Epidermophyton floccosum* Hartz, *Cladosporium mansoni* Cast., *Aleurisma castellanii* Pinoy (*Acladium castellanii*).

These distilled water tubes were inoculated from dextrose agar cultures, care being taken that particles of the dextrose medium were not transferred to the liquid. The tubes were sealed at the flame and kept at room temperature until July 10th 1939. On that day the tubes were opened, breaking the necks after filing and after shaking inoculations were made from each tube into dextrose agar medium. Growth developed in all the dextrose agar tubes within the normal time and the macroscopic appearances of the cultures were normal.

The *Candidae* were passed through the usual carbohydrates (dextrose, levulose, mannose, maltose, galactose, lactose, inulin). *C. albicans* var. *pinoyi* produced gas-fermentation in dextrose, mannose, fructose, and maltose; *C. krusei* in glucose, fructose and mannose; *C. tropicalis* in dextrose, fructose, mannose, maltose, galactose, saccharose; *C. macedoniensis* in glucose, fructose, mannose, galactose, saccharose and inulin; *C.*

pseudotropicalis in dextrose, fructose, mannose, galactose, saccharose, and lactose. The microscopical characters appeared to be unchanged. The strain of *Epidermophyton floccosum* inoculated into distilled water was the old laboratory strain which had become partially pleomorphic several years previously, being fluffy but still showing a certain amount of characteristic canary-yellow colour. The cultures made on dextrose agar after twelve months maintenance of the fungus in distilled water, showed the same partial pleomorphism, with some characteristic yellow colour present.

From the amount of sediment in the inoculated distilled water tubes the impression was obtained that several of the fungi must have grown slightly. This was certainly the case with *Cladosporium mansoni* and some species of *Candida*.

Since the war the experiment has been repeated more than once using the fungi mentioned above, and in addition the following: *Sporotrichum anglicum* Cast., *Glenospora lanuginosa* Cast., *Trichophyton rubrum* Cast., and other species of *Trichophyton* and *Microsporum*; also *Coccidioides immitis* Rixford and Gilchrist, strain *metaeuropeus* Cast., *Blastomyces dermatitidis* Gilchrist and Stokes, strain *tulanensis* Cast., *Cryptococcus neoformans* Sanfelice, *C. neoformans* strain *hondurianus* Cast., *C. ater* Cast. (this last was inoculated in sterile distilled water in May 1959). The result have been the same. After 12 months, all the fungi were found alive, and grew quite well when inoculated into dextrose agar, producing colonies exactly like the original ones, and the biochemical characters had remained the same.

In recent years the technique has been simplified. Ordinary tubes containing 6.8 or 10 ml. of sterile distilled water (boiled on three consecutive days or autoclaved) and plugged with cotton wool like ordinary tubes of broth are used. They are inoculated with a « large » inoculum and kept in the laboratory at room temperature or in the incubator at 18-22° C.

(In hot countries, if kept at the temperature of the room, it is advisable to use rubber caps to prevent too much evaporation and loss of liquid). When using a large inoculum, it is practically unavoidable to transfer a small portion of the dextrose agar to the tube of distilled water, but the amount of dextrose so added is so minute a quantity that it is not likely that it can sensibly influence the growth of the fungus or facilitate the development of pleomorphism.

The above experiments have led to a very simple procedure for maintaining pathogenic fungi and especially dermatophytes, in mycological collections, that is: cultivation is sterile distilled water. The tubes of sterile distilled water are inoculated with the fungi and left at room temperature for twelve months. They may be sealed in a flame or merely plugged with cotton wool. Transplantations are made from them after one year on to dextrose agar to see whether the fungi are alive and have maintained their original characters. From these dextrose agar culture a new series of distilled water tubes are inoculated and left at room temperature (or at 18-20°C in the incubator) for a year, when dextrose agar cultures are again made. From these, new series of sterile distilled water tubes are inoculated. This method does away with the necessity of frequent subculturing and makes unnecessary the use of lysolitic procedures, which often are much less successful with mycetes than with bacteria, some mycetes, e.g. *Cladosporium mansoni* frequently dying out in the process. Another practical advantage of the method is that it largely seems to prevent pleomorphism: none of the dermatophytes experimented with so far have become pleomorphic during their long sojourn in sterile distilled water. This procedure apparantly prevents pleomorphism, but of course does not cure it once it has developed: a pleomorphic strain inoculated into sterile distilled water remains pleomorphic.

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