FURTHER RESEARCHES ON THE LONG VIABILITY AND GROWTH OF MANY PATHOGENIC FUNGI AND SOME BACTERIA IN STERILE DISTILLED WATER

by

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(with 3 figs.)

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In the Journal of Tropical Medicine & Hygiene, August Ist, 1939, I published a paper on the long viability — over a year — of a number of pathogenic fungi in sterile distilled water, without ever changing the water or adding any substance of whatever nature to it.

I quote a portion of the paper *verbatim* without altering the taxonomic nomenclature used at that time, but adding in square brackets the nomenclature used at present.

"On July 5th, 1938, thirteen tubes of sterile distilled water were inoculated with the following fungi (each with one fungus):

Monilia [*Candida*]:— *Monilia krusei* CAST. [*Candida krusei*]; *Monilia pinoyi* CAST. [*Candida albicans* var. *pinoyi*]; *Monilia tropicalis* CAST. [*Candida tropicalis*]; *Monilia pseudotropicalis* CAST. [*Candida pseudotropicalis*]; *Monilia macedoniensis* CAST. [*Candida macedoniensis* var. *macedoniensoides*].

Geotrichum:— *Geotrichum rotundatum* CAST., *Geotrichum matalense* CAST. *Geotrichum asteroides* CAST., *Geotrichum rugosum* CAST.

Miscellaneous:— *Epidermophyton floccosum* HARTZ, *Cladosporium mansoni* CAST., [*Aureobasidium mansoni*], *Aleurisma castellanii* PINOY (= *Acladium castellanii*), *Actinomyces sylvodorifera* CAST. [*Streptomyces sylvodorifera*] (no longer considered a fungus).

The distilled water tubes were inoculated from dextrose agar cultures, taking care not to transfer to the liquid particles of the dextrose agar. The tubes were sealed at the flame and kept at the temperature of the room in the mycological laboratories of the London School of Hygiene and Tropical Medicine until July 10th, 1939. On that day the tubes were opened, breaking the necks after filing, and after shaking inoculations were made in dextrose agar tubes.

Growth developed in all the dextrose agar tubes within the normal

time and the *Moniliae* [*Candidae*] were passed through the usual carbohydrates (dextrose, laevulose, maltose, galactose, saccharose, lactose, inulin) and gave the typical specific gasfermentation reactions. The strain *Monilia pinoyi* [*Candida albicans* var. *pinoyi*] produced gasfermentation in dextrose, laevulose and maltose; *Monilia* [*Candida*] *krusei* in dextrose and laevulose; *Monilia* [*Candida*] *tropicalis* in dextrose, laevulose, maltose, galactose, saccharose; *Monilia* [*Candida*] *macedoniensis* var. *macedoniensoides* in dextrose, laevulose, galactose, saccharose and inulin; *Monilia* [*Candida*] *pseudotropicalis* in dextrose, laevulose, galactose, saccharose and lactose. An investigation of the microscopical characters is being carried out at the present time. (No change was found).

The strain of *Epidermophyton floccosum* inoculated into distilled water on July 5th, 1938, was the old laboratory strain which became partially pleomorphic several years ago, being fluffy but still showing a certain amount of the characteristic canary-yellow colour. The culture made on dextrose agar after twelve months in distilled water showed the same partial pleomorphism with the same characteristic yellow colour present.

From the amount of sediment in the inoculated distilled water tubes one had the impression that several of the fungi must have slightly grown (*Geotrichum matalense*, *Geotrichum asteroides*, *Monilia* [*Candida*] *tropicalis*). This is certainly the case with regard to *Cladosporium* [*Aureobasidium*] *mansoni*."

Further Researches

Simplification of the Technique. — Since 1939 I have repeated the experiment several times with the fungi mentioned above and certain other pathogenic fungi, simplifying the original technique which entailed the closing of the inoculated tubes at the flame. I simply use ordinary tubes containing 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 or other media. They are inoculated with a large inoculum and kept in the laboratory at the temperature of the room (in hot countries it is advisable to use rubbercaps to prevent too much evaporation and loss of liquid). When using a large inoculum it is almost impossible with filamentous fungi to prevent a bit of the dextrose agar from the stock dextrose agar culture being transferred to the tube of sterile distilled water, but the amount of dextrose so added to the water is of such minute quantity that it is not likely to influence sensibly the growth of the fungus or facilitate the development of pleomorphism.

Species of Fungi Experimented with. — In addition to the fungi mentioned in the quoted paper, I have investigated the following ones: *Sporotrichum anglicum* Cast., *Glenospora lanuginosa* Cast., *Trichophyton rubrum* Cast., and other species of *Trichophyton* and *Microsporum*; also *Coccidioides immitis* Rixford & Gilchrist strain

metaeuropeus CAST., *Blastomyces dermatitidis* GILCHRIST & STOKES strain *tulanensis* CAST., *Cryptococcus neoformans* SANFELICE strain *hondurianus* CAST., *Cryptococcus ater* CAST.

The results have always been the same: after twelve months' permanence in sterile distilled water at the temperature of the room all the fungi were found alive and grew quite well when inoculated on dextrose agar and other solid media. Moreover it was found that they would all remain viable for a further very long period of time, up to two years and longer, if the water is not allowed to evaporate completely (using rubber-caps etc.), — and perhaps indefinitely if the evaporated portion of water is replaced with new sterile distilled water at yearly interval.

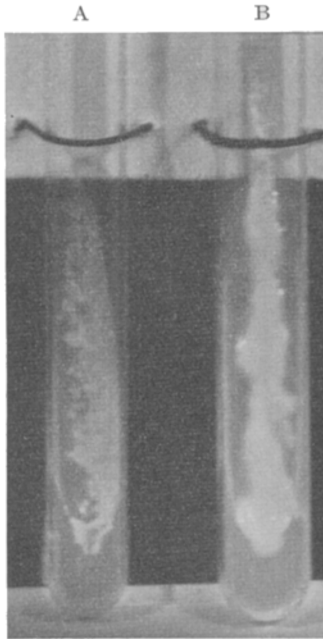

Fig. 1 (A) Five-days-old dextrose agar culrure made from distilled water culture of *Candida krusei* 24 months old.
(B) Five-days-old dextrose-agar culture made from distilled water culture of *Cryptococcus neoformans* 24 months old.

Subculturing, with large inocula, from distilled water cultures into sterile distilled water tubes every three or six or twelve months was also found successful with the fungi I experimented with, namely *Cryptococcus neoformans* SANFELICE, *Candida albicans* ROBIN var. *pinoyi* CAST., *Candida krusei* CAST., *Candida tropicalis* CAST., *Candida guillermondi* CAST., *Geotrichum matalense* CAST., *Aureobasidium mansoni* CAST. Viability appeared to remain intact, but actual multi-

plication is much less evident. It must be kept in mind of course that with some exceptions such as *Aureobasidium mansoni* (*Cladosporium mansoni*) and certain yeasts distilled water cultures of pathogenic fungi are never abundant or profuse.

Sterile Distilled Water Method for Maintenance of Fungal Strains in Mycological Collections. — The above experiments have led me to recommend the 'distilled water cultivation' method as a very simple procedure for maintaining pathogenic fungi, especially dermatophytes and yeasts, in mycological collections. Sterile distilled water tubes are inoculated with the fungi and left alone at room temperature for twelve months, when transplantations are made on to glu-

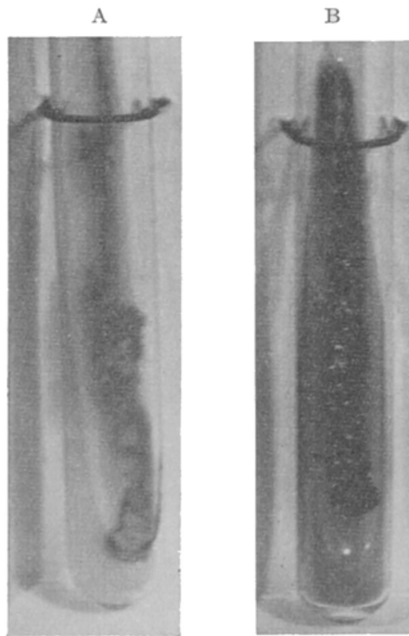

Fig. 2 (A) Three weeks-old dextrose agar culture made from distilled water culture of *Aureobasidium mansoni* (*Cladosporium mansoni*) 24 months old.
(B) Four-weeks-old dextrose agar culture made from distilled water culture of *Cryptococcus ater* 24 months old

cose agar to see whether the fungi are alive and have maintained their original characters. From these glucose agar cultures a new series of distilled water tubes are inoculated and left at room temperature for another year, and then a new series of sterile distilled water tubes are inoculated, and so on. This method eliminates the necessity of frequent subculturing and makes unnecessary the use of lysolitic procedures, which in my experience are much less successful with mycetes than with bacteria, some mycetes such as *Aureobasidium*

mansoni (*Cladosporium mansoni*) dying out in the process. Another practical advantage of the method is that it seems to prevent, to a great extent, pleomorphism, but of course it does not cure it once it has developed: a pleomorphic strain inoculated into sterile distilled water will still remain pleomorphic. I have recently noted that pleomorphism does not generally occur in tubes in which, owing to the use of rubber caps or having been sealed at the flame, very little or no evaporation takes place, while in tubes the liquid of which has evaporated to a great extent, pleomorphism may develop.

Viability of Certain Bacteria in Sterile Distilled Water. — In my lecture *Miscellaneous Mycological Notes*, given before the New York Academy of Sciences on May 27th, 1960, I mentioned that not only fungi but also certain bacteria, as for instance *Salmonella typhosa*, could remain alive and multiply for over a year in sterile distilled water. My researches on the maintenance of bacteria in sterile distilled water have been much less extensive than those on mycetes. I can state, however, that a certain number of bacteria, especially of the family *Enterobacteriaceae* have been found capable of long maintenance (over a year) in sterile distilled water and may multiply in it. At the present time I have viable distilled water cultures over a year old, of the following bacteria among others: *Salmonella typhosa, Salmonella paratyphi, Salmonella schottmuelleri, Salmonella asiatica, Morganella columbensis, Corynebacterium mycetoides.* The technique is the same as for fungi. Sterile distilled water tubes each containing 8—10 ml are inoculated with a large bacterial inoculum and plugged with cotton wool (or sealed at the flame) and left at the temperature of the laboratory for twelve months (rubber caps should be used in hot countries.) Subcultures on ordinary solid media are then made to see whether the organisms are alive and have maintained their characters.

Summary and Conclusions

The old researches carried out by me at the London School of Hygiene and Tropical Medicine in 1938—39, published in the Journal of Tropical Medicine & Hygiene, August, 1939, demonstrating the long viability — over a year — of many pathogenic fungi in sterile distilled water have been confirmed and amplified. It has been found, also, that a certain number of bacteria, especially of the family *Enterobacteriaceae* are capable of remaining alive and at times multiplying in sterile distilled water for over twelve months and much longer.

2) The pathogenic fungi mentioned in this paper remain viable in distilled water for periods of time far exceeding a year if care be taken not to allow the distilled water to evaporate completely (using rubber caps), — and it would almost appear — indefinitely, if the evaporated portion is replaced at long intervals (yearly) with new sterile distilled water.

Subculturing distilled water cultures direct into sterile distilled water at long intervals (yearly) seems also to be successful with the strains I have mentioned.

3) A simple method for maintaining pathogenic fungi in mycological collections has been devised and introduced by me — the 'sterile distilled water culture method', requiring subculturing only once a year. The reliability of the method with regard to many pathogenic fungi has been confirmed by CASTEGNETTA and MUNGELLUZZI (Arch. It. Scien. Mediche Tropicali & Parasit., Feb. 1962) and its simplicity and usefulness have been emphasied by the well known authority on mycology, TIBOR BENEDEK, who in his recent paper (Mycopathologia et Mycologia Applicata, vol. **18**, 3, 255—261) has written: "CASTELLANI's water culture method for microscopic fungi was re-examined and confirmed in its every detail. It is an ideal method for, at least, the smaller culture collections, in order to avoid continuous short-term subculturing".

In this paper, as in my previous ones on the same subject, I have limited myself to relating facts and observations without any attempt at formulating theoretical explanations. The viability of many pathogenic fungi and some bacteria in sterile distilled water may be explained up to a point by the nutritional and other reserves carried by the cells forming the first inoculum, but are such reserves sufficient to explain the viability and capability of growth in distilled water for periods of time which may extend to well over a year? This seems to me to be extremely doubtful. I hope that my work may act as a small stimulus to the active research now going on in many scientific centers on certain biologic subjects of broad intellectual interest: the really essential requirements of fungi and bacteria for life and growth, endogenous metabolism and what has been called energy of maintenance.

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