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LONG TERM MAINTENANCE AND CULTIVATION OF THE COMMON PATHOGENIC FUNGI OF MAN IN STERILE DISTILLED WATER

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SVMMARIVM — Communiores hominis fungi pathogeni — praeter Actinomyces, qui tamen in fungis iam haberi non solet — et vivere et numero augeri, per annum saltem, possunt si in aqua destillata sterili ponantur; quod si in evaporatae aquae locum nova singulis annis subrogetur, et phialae sint omnino clausae, ii fungi per tempus tam longum, ut paene indefinitum videatur, vivere possunt.

Nearly three decades ago I gave an account in the « Journal of Tropical Medicine and Hygiene » (*42*, 255, 1939) of an experiment carried out by me in the Laboratories of the London School of Hygiene and Tropical Medicine where I was holding at the time the position of Director of Mycology and Mycoses.

ORIGINAL EXPERIMENT

On July 5th, 1938, twelve tubes of sterile distilled water were inoculated with the following fungi: *Candida krusei* Cast.,

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C. *albicans* Robin var. *pinoyi* Cast., *C. tropicalis* Cast., *C. pseudotropicalis* Cast., *C. macedoniensis* Cast., *Geotrichum rotundatum* Cast., *G. matalense* Cast., *G. asteroides.*, Cast., *G. rugosum* Cast., *Epidermophyton flocossum* Hartz, *Cladosporium mansoni* Cast., *Aleurisma* (*Acladium*) *castellanii* Pinoy.

The tubes of distilled water were inoculated from glucose agar cultures, care being taken that particles of the glucose were not transferred to the liquid. The tubes were sealed at the flame and kept at room temperature until July 10th, 1939, a period of one year and five days. The tubes were then opened and after shaking, inoculations were made from each tube into glucose agar. Growth developed in all the glucose agar tubes within the normal time and the macroscopic appearance of the cultures was normal.

The *Candidae* were passed through the series of Carbohydrates in use at that time, namely, glucose, leavulose, mannose, maltose, galactose, saccharose, lactose and inulin. The fermentation characters had not undergone any change.

The strain of *E. flocossum* inoculated into distilled water was an old laboratory strain which had become partially pleomorphic several years previously, being fluffy bnt still showing a certain amount of characteristic canary-yellow colour. The cultures which were made on glucose agar after twelve months' maintenance in distilled water, showed the same partial pleomorphism with some characteristic yellow colour present.

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

Since then the experiment has been repeated many times using the fungi mentioned above and in addition practically all the other cultivable fungi producing disease in man, among which: *Trichophyton rubrum* Cast., and a number of other species of *Trichophyton* and *Microsporon* including *Tr. concentricum* Blanchard var. *tropicale* Cast., also *Coccocidiodes*

immitis Rixford and Gilchrist, strain *metaeuropeus* Cast., *Blastomyces dermatitidis* Gilchrist and Stokes *strain tulanensis* Cast., *Crytococcus neoformans* Sanfelice, *C. neoformans* Sanfelice var. *hondurianus* Cast., *C. ater* Cast., *C. genitalis* Cast. The results have been constantly the same. After twelve months all the fungi were alive and grew quite well on glucose agar, producing colonies exactly like the original ones, having the same morphological and biochemical characters; moreover, it was found that they remained viable in water far longer than a year, in fact it would appear this they would live indefinitely if the evaporated water were replaced yearly. The very simple original procedure has been rendered even simpler in recent years by discarding the sealing of the inoculated tubes at the flame and using cotton wool plugs.

PRESENT-DAY TECHNIQUE

Ordinary tubes (plugged with cotton wool) containing 6 to 10 ml of sterile distilled water are sterilized by autoclaving, or by boiling on three consecutive days, and then stored like other liquid media until required for use.

The inoculation of the various fungi is carried out in exactly the same manner as if the medium were peptone water or broth using a fairly large inoculum. The inoculated tubes are kept at room temperature taking the precaution in hot countries of covering the cotton wool plugs with rubber caps or applying slaling to prevent too much evaporation. After a year, subcultures are made on glucose agar to see whether the fungi are still alive and whether they maintain their characteristics. From these cultures a new series of distilled water tubes is inoculated and kept at room temperature for a year and then they again tested for viability, thereafter new sterile distilled water tubes are inoculated and so on.

Certain fungi produce on glucose agar, hard deep-rooted colonies and when a fairly large inoculum is used it is almost impossible to prevent the transfer of a particle of glucose medium to the tube of distilled water, but the amount of glucose so added is so minute that it is not likely to influence sensibly the growth of the fungus or facilitate the development of pleomorphism. Moreover, the inoculum may be taken from maintenance agar cultures instead of glucose agar culture.

If the tubes are sealed at the flame or ermetically closed in some other way thereby preventing evaporation, up the water the fungi seem to remain alive for many years, almost indefinitely without treeh changing the water.

Modifications of the method

The method can be modified in many ways. For instance at the end of each year the evaporated water is simply replaced by fresh sterile distilled water without first making glucose agar or maintenance agar cultures. The yearly subculturing can also be done direct from distilled water cultures to sterile distilled water tubes.

Another more important variation is what I have called the solid agar-distilled water method: to ordinary glucose agar or maintenance agar slopes, 1.50 to 2 ml of sterile distilled water is added or an amount sufficient to keep the lowest one third or the lowest half of the surface of the slope covered by water. In this way one can follow and study daily the development of the organism in both media. Unfortunately, however, for various reasons (evaporation, absorption by solid medium, etc.) the water frequently disappears almost completely and has to be replaced at fairly frequent intervals.

There cannot be any doubt that humidity is favourable to most fungi while desiccation is one of their worst enemies. It

has occasionally been possible to revive dried up cultures several months old by simply adding some sterile distilled water to the tubes.

Advantages and usefulness of the method

The method does away with the tedious and time-consuming monthly or fortnightly subculturing and renders unnecessary lysolitic procedures which in my experience are much less successful with fungi than with bacteria.

Another advantage when applied to the dermatophytes is that it prevents at least, partially the development of pleo- that it prevents, at least partially, the development of pleomorphic strain maintained in sterile distilled water will remain pleomorphic.

Sabouraud used to say that the poorer the medium the less the pleomorphism, and for this reason he introduced his maintenance agar medium which is agar prepared with peptone water instead of broth and with no addition of sugar. Can one find a poorer medium than sterile distilled water?

Certain fungi, fortunately few, after prolonged culturing on agar media seem to develop a tendency to die out seddenly without any apparent cause. Last year all the cultures of *Cladosporium mansoni* in a continental laboratory suddenly became non-viable although they had been regularly subcultured monthly. The same occurred more recently in a British laboratory. On both occasions the situation was saved by having recourse to the water cultures of the fungus kept in a Portuguese laboratory. They were quite viable.

The routine use of the method has brought to light the not quite exceptional presence of concommitant bacteria in supposedly pure fungal agar cultures. These bacteria, usually cocci, are present in extremely small numbers and seem to

remain dormant while the fungus is cultivated on agar, but very gradually revive and very slowly multiply when the fungus is cultured in distilled water (or peptone water or broth). The addition of one per cent boric acid or I:2000 salicylic acid or I:20,000 mercury perchloride water will kill the concommitant bacteria without interfering sensibly with the development and viability of the fungus. (Antibiotics can be used of course). This subject will be fully discussed in a future paper.

Application of the method to some bacteria

In a lecture given by me in May 1960 at the New York Academy of Sciences and reported in *extenso* in the Annales of that Academy (vol. 93, art. 5, pp. 147-266, Feb. 1962), attention was called to the fact that not only practically all pathogenic fungi of man are viable and capable of growth in sterile distilled water for over a year. But also a fairly large number of bacteria are capable of doing so, especially bacteria of the family *Enterobacteriaceae* (*Salmonella typhosa, S. paratyphosa, S. Schottmullieri, S. asiatica Morganella columbensis, Proteus morgani, Cloaca cloacae* etc.) and also members of several other families.

Summary and conclusions

My researches initiated in 1938-39 and continued at intervals until the present day appear to have demonstrated that practically all the cultivable fungi pathogenic to man excluding some Actinomycetes (they are no longer considered to be fungi) remain viable and capable of growing in sterile distilled water for at least a year, and, it would appear almost indefinitely, if the evaporated water is replaced annually or apparently even

if the water is not changed provided the tube is hermetically sealed, at the flame or in some other way, thereby inhibiting, water evaporation.

Based on these results a simple method has been devised requiring a single yearly subculture for the maintenance of pathogenic fungi in mycological collections. The reliability and usefullness of the method have been emphasized by BENEDEK, MUNGELLUZZI, CASTAGNETTA and others. BENEDEK has written in *Mycopathologia et Mycologia Applicata*, 1967, *17*, 225: « Castellani's water cultivation method for microscopic fungi was re-examined and confirmed in every detail. It is an ideal method for at least the smaller collections to avoid continuous short term subculturing »

It can be repeated that apparently there is no need of changing the water if the tubes have been hermetically sealed, the fungi remaining viable for years when kept at the temperature of the room or in the refrigerator (4 to 11° C.)

If the tubes of sterile distilled water containing the fungi are kept hermetically sealed as for instance by sealing at the flame, the fungi seem to remain alive for years without changing the water annually.

The method when applied to the dermatophytes is useful in preventing at least partially the development of pleomorphism. It is useful also when applied to those fungi, fortunately few, which after prolonged cultivation on agar media seem to develop a tendency to die out suddenly without any apparent cause.

In addition to the mycetes the method can be applied to a certain number of bacteria including such pathogens as the organism of typhoid and paratyphoid.

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