A New Staining Solution for the Morphological Studies of Fungi and *Prototheca*

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ABSTRACT. A new staining solution named "PHOL" is described for studying the morphology of clinical and environmental isolates of fungi and *Prototheca*. In total 69 isolates were used for assessing the efficacy of this newly formulated stain. The solution has shown good ability to stain all 62 isolates of fungi and 7 of *Prototheca*, and thus helped in the identification of the organisms. The new solution has the potential to stain the young as well as old isolates of fungi and *Prototheca*. It is emphasized that a wider application of this newly introduced inexpensive and safe staining solution will help the mycologists to study the morphological characteristics of yeast, molds, and *Prototheca* obtained from man, animals and environment.—**KEY WORDS:** fungus, morphology, *Prototheca*, staining solution.

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Numerous stains are employed by the microbiologists for the identification of infectious agents which cause serious illnesses and fatal diseases in man and animals [1]. Among these, lactophenol cotton blue (LPCB) is the most commonly used stain for studying the detailed morphological characters of a fungal isolate [3–6]. However, our experience for more than two decades showed that some of the dermatophytes were not easily stained by the conventional LPCB. This initiated us to undertake a pilot project to develop a suitable staining solution which can impart good staining reaction to dermatophytes as well as other fungi.

In this paper, we describe the efficacy of a new staining solution to study the morphology of fungi and *Prototheca*.

MATERIALS AND METHODS

Organism: A total of 69 isolates (commonly associated with various clinical disorders of men and animals) which comprised of 62 fungi (41 yeasts and 21 molds), and 7 *Prototheca* (the culture of which resembles

yeasts) were used for the present study. These cultures were maintained in the laboratory in active form by periodical passages on Sabouraud's dextrose agar with incubation at room temperature. For the present experiment, each isolate was grown on modified Sabouraud's medium [2] at 25°C for 3 to 14 days.

Stain: The new staining solution (PHOL) has the following composition:

5 ml of 4% aqueous solution of 35% formaldehyde, 3 ml of glycerol, and 0.3 ml of 3% aqueous solution of methylene blue. All these ingredients were mixed together in a clean, sterilized glass stoppered bottle and kept at room temperature for further use.

Experiment: A small loopful of the culture was added to a drop of this new staining solution on a clean, dust and grease-free glass slide. The examination was made in 20X and 40X with a microscope after putting a clean coverslip. For a comparison, LPCB was used as a standard stain to distinguish if at all any change in the morphology of the organism has occurred due to this new solution.

RESULTS

All the 69 isolates of fungi and *Prototheca* were subjected to detailed microscopic examination in LPCB as well as PHOL stain. Each wet mount was examined within 5–10 minutes of its preparation; and the staining reaction was graded as poor, good and excellent. The details of the organisms examined for their morphological characteristics in PHOL and LPCB staining solutions are summarized in Table 1. It is obvious from the microscopic findings that the new solution gave consistent results comparable to a well established LPCB stain, as no

difference in the morphology could be noticed in any of the fungal and *Prototheca* strains (Figs. 1–8). However, PHOL solution resulted in better staining to the isolates of *Candida* spp., *Prototheca* spp., *Microsporum* spp., and *Trichophyton* spp. The new stain has a potential to penetrate deep into the organisms and thus imparts good bluish colour which helps in the identification of the molds, yeasts and *Prototheca*. Furthermore, none of the 69 isolates showed any difference in their morphological changes when examined after 12–18 hrs of the preparation. However, a few cells of *C. neoformans* and *T. beigeli* showed light purple colour in PHOL solution.

Table 1. Comparison of the efficacy of LPCB and PHOL mounting fluids in studying the morphology of fungi and algae

Name of organism	No of strains tested	Microscopic results	
		LPCB	PHOL
FUNGI			
<i>Cryptococcus neoformans</i>	13	+ ^{b)}	++ ^{c)}
<i>Candida tropicalis</i>	8	++	++
<i>Candida albicans</i>	3	+	++
<i>Candida glabrata</i>	1	+	+
<i>Candida guilliermondii</i>	1	+	+
<i>Candida kefyr</i>	1	++	++
<i>Candida krusei</i>	1	++	++
<i>Candida parapsilosis</i>	1	+	+
<i>Candida</i> spp.	6	+	+
<i>Rhodotorula</i> spp.	4	++	++
<i>Trichosporon beigelii</i>	2	++	++
<i>Aspergillus terreus</i>	3	+	++
<i>Aspergillus flavus</i>	2	+	+
<i>Aspergillus fumigatus</i>	2	+	+
<i>Aspergillus niger</i>	2	+	+
<i>Microsporum gypseum</i>	2	++	++
<i>Microsporum canis</i>	1	+	++
<i>Microsporum vanbreuseghemii</i>	2	— ^{a)}	++
<i>Trichophyton ajelloi</i>	2	—	++
<i>Trichophyton mentagrophytes</i>	2	+	++
<i>Trichophyton rubrum</i>	1	+	++
<i>Trichophyton simii</i>	2	+	++
ALGAE			
<i>Prototheca zopfii</i>	3	+	+
<i>Prototheca wickerhamii</i>	4	++	+

a) Poor staining, b) Good staining, c) Excellent staining.

Fig. 1. A clinical isolate of *C. albicans* in LPCB stain, $\times 400$.

Fig. 2. The same isolate of *C. albicans* in PHOL stain, $\times 400$.

Fig. 3. LPCB mount of a clinical isolate of *T. beigeli*, $\times 400$.

Fig. 4. PHOL mount of the same isolate of *T. beigeli*, $\times 400$.

Fig. 5. A clinical isolate of *M. canis* in LPCB stain, $\times 400$.

Fig. 6. The same isolate of *M. canis* in PHOL stain, $\times 400$.

Fig. 7. LPCB mount of a clinical isolate of *P. zopfii*, $\times 400$.

Fig. 8. PHOL mount of the same isolate of *P. zopfii*, $\times 400$.

DISCUSSION

The findings of this preliminary experiment clearly indicated that PHOL stain is a good substitute to LPCB and can be successfully employed by the microbiologists to study the detailed morphology of fungi as well as *Prototheca*. It is pertinent to mention that all 69 strains of various organisms showed similar morphology in PHOL as observed in well established LPCB stain [1-3]. Moreover, the penetrating action of the formalin helped to study the cytoplasmic structure and nucleus of the organism in a better way. It is noteworthy that old cultures (35-50 days) of the fungi and *Prototheca* were easily stained by this new method. Also the slide in PHOL stain does not get dry and can be preserved for sometime (12-24 hrs) as experienced with LPCB. Further, no difference was noticed in the microscopic morphology of clinical and environmental isolates in the new stain.

Our experience showed that macroconidia of *T. ajelloi* and *M. vanbreuseghemii* usually did not stain well with LPCB. It is interesting to note that all the dermatophytes including *M. vanbreuseghemii* and *T. ajelloi* are easily stained by this new technique. It has also been observed that some mycologists treat the fungal culture

with alcohol before staining with the LPCB to obtain quick results. However, no such pretreatment is required in PHOL solution as the organisms are rapidly stained. The development of light purple colour in some cells of *C. neoformans* and *T. beigeli* is due to the presence of methylene blue in PHOL solution. However, the purple colour does not interfere in the identification of both fungi. Presently, no plausible explanation can be given why methylene blue imparts light purple colour to the cells of both the yeasts.

As formalin has a tendency to slide down, one should be prompt in preparing the wet mounts. However, the addition of 3 ml of glycerol to 5 ml of formalin solution, helped to stop the moving tendency of the fluid to a great extent.

In vitro studies carried out by one of us (M. Pal, Personal communication) has revealed the fungicidal action of 4% formalin against many yeasts and molds. Therefore, laboratory staff may enjoy safety against an accidental occupational hazard while working with potential and opportunistic fungal pathogens.

The new stain is relatively very cheap, easily available, simple to prepare, quite stable at room temperature besides great safety to the laboratory workers. It may be

concluded that a wider use of this newly formulated solution in microbial diagnostic laboratories in either human or animal field is expected because of its good staining ability as well as its economy and safety.

The name PHOL to this new stain is derived from the initials of surname of each author.

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要 約

真菌および藻類の形態学的観察のための染色液：Pal, M.・長谷川篤彦・小野憲一郎¹⁾・Lee, C.W. (東京大学農学部獣医内科学教室, ¹⁾ 獣医臨床病理学教室)——臨床材料および自然界から分離された真菌(22種62株)と藻類(2種7株)の形態学的観察に新たに調製した染色液(PHOL)を用い, その有用性を検討した. PHOLはホルムアルデヒド(35%)の4%水溶液5 ml, グリセロール3 ml, メチレンブルー3%水溶液0.3 mlからなり, その染色性は従来使用されているラクトフェノール・コットンブルー染色液と比較して劣るものではないことが確認された.