

# Comparison of Microscopic Morphology of Fungi Using Lactophenol Cotton Blue (LPCB), Iodine Glycerol and Congo Red Formaldehyde Staining

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Sir,

Although fungi were considered as innocuous environmental saprophyte, the significance of fungi as human pathogen is on the rise [1]. Hence, laboratory diagnosis of fungal infections has critical role in patient care. In this prospective study, which was carried out from March to September, 2013, we have evaluated the performance of iodine-glycerol in comparison to lactophenol cotton blue (LPCB) and Congo-red formaldehyde as an alternative method for identification of filamentous fungi.

A total of 180 clinical samples, obtained from skin, hair, nail, sputum, broncho-alveolar lavage, corneal scrapings and tissue of various inpatient and outpatient departments of Mahatma Gandhi Medical College & Research Institute, Pondicherry, India were processed for fungi and included in this study.

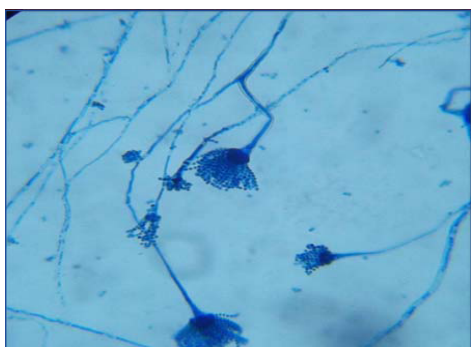
Direct microscopic examination was performed using potassium hydroxide (KOH) iodine glycerol and Congo red formaldehyde wet mounts [Table/Fig-1-3]. Culture was done in duplicate in SDA with and without cycloheximide at 25°C and 37°C incubation.

Tease mount and slide cultures of 20 standard fungal strains (belonging to the genus *Aspergillus*, *Penicillium*, *Fusarium*, *Curvularia*, *Mucor*, *Rhizopus*, *Syncephalastrum*, *Trichophyton*, *Epidermophyton* and *Microsporum*) and fungal clinical isolates grown on culture were stained by Congo red formaldehyde, LPCB and iodine-glycerol in an attempt to compare their staining characteristics.

Iodine glycerol reagent was prepared in-house by addition of glycerol (0.25%) in iodine solution as described by Vignesh et al., [2]. Whereas for Congo red formaldehyde stain, 10% formaldehyde solution was diluted to 1% and Congo red was added to achieve 0.1% final concentration at pH 7.1 [3]. Wet preparations of Congo red formaldehyde were examined under fluorescence microscope. Fungal culture was considered as the gold standard test and sensitivity, specificity, positive predictive value and negative predictive value were calculated.

Total 32 fungal isolates were recovered in culture [Table/Fig-4]. The performance of KOH, iodine glycerol and Congo red formaldehyde wet mounts in comparison to culture are expressed in terms of sensitivity, specificity, positive predictive value and negative predictive value and detailed in [Table/Fig-5]. Direct microscopic detection of fungal pathogens has immense value in routine mycology workup. Especially in case of opportunistic mycoses, isolates grown on culture often require direct microscopic confirmation to rule out the possibility of laboratory contamination [4]. KOH, in various concentrations, has been employed as a keratolytic agent to demonstrate fungi in tissue [5]. However, it does not stain fungal elements. Consequently, delicate semi-transparent fungal elements escape detection [6-8]. On the other hand, collagen fibers, tissue matrix, lipid vesicles, air bubbles, and textile fibers are often difficult to distinguish from fungi [6]. Several authors had reported low sensitivity and specificity of KOH mounts [6-8]. Saxena et al., reported 68% sensitivity and 40% specificity [6]. In our study, the sensitivity and specificity of KOH was 65.6% and 93.9% respectively.

Fluorescent dyes which bind specifically to fungal cell wall have essential role in mycology. Calcofluor white was traditionally used as a fluorescent brightener in paper and textile industries [3, 5], has been extensively used to study cell walls of fungi and bacteria. The use of other fluorescent agents in mycology have been reported infrequently. Congo red is a diazo compound which is commonly used to stain amyloid in tissue sections [3]. Pertaining to its high affinity for cellulose or chitin, Congo red also permits the detection of fungal elements in tissue. Although Congo red is cost-effective compared to Calcofluor white, its use as fungal stain is still unconventional [3,9]. Congo red in conjunction with glutaraldehyde or formaldehyde, as fungicidal agent, had been reported to be useful for rapid detection fungi in both clinical specimens and fungal culture preparations [3]. We found that yeast cells and both septate and aseptate hyphae in clinical specimens had bright orange fluorescence. In case of culture preparations of filamentous fungi,



**[Table/Fig-1]:** Tease mount preparation of fungal culture using LPCB showing penicillium sp **[Table/Fig-2]:** Formalin-Congo red-stained tease mount preparations of zygomycetes showing aseptate hyphae **[Table/Fig-3]:** Wet preparation using iodine glycerol showing macroconidia of Microsporum spp

Fungal isolate	Number	Percentage
<i>Candida albicans</i>	8	4.4%
Non-albicans <i>Candida</i> species	11	6.1%
<i>Aspergillus fumigatus</i>	4	2.2%
<i>Aspergillus flavus</i>	2	1.1%
<i>Aspergillus niger</i>	1	0.5%
<i>Penicillium</i> species	1	0.5%
<i>Fusarium</i> species	1	0.5%
<i>Mucor</i> species	2	1.1%
<i>Rhizopus</i> species	1	0.5%
<i>Trichophyton mentagrophytes</i>	1	0.5%

[Table/Fig-4]: Fungal isolate recovered in culture from clinical samples

the cell walls, cross-walls of hyphae, as well as the hyphal tips and rhizoids were visible. However, the conidia of molds, *Aspergillus*, *Penicillium* and *Fusarium* species in particular, were indistinct and often not visible under fluorescent microscope. Slifkin et al., found good staining result of Congo red for a variety of yeasts and molds except the cell wall of *Phialophora* species and the capsule of *Cryptococcus neoformans* which were poorly stained [3]. In another study, *Candida* and *Histoplasma* isolates were found Congo red negative unlike *Pityrosporum* and *Blastomyces* isolates [9].

Although KOH and Congo red are easily available and inexpensive reagent, both may have potential health hazard. As per material safety data sheets, formaldehyde used in Congo red preparations is cytotoxic and carcinogenic [10]. Whereas, KOH solutions with concentrations higher than 2% are corrosive [11]. In a recent study, better microscopic morphology of fungi was obtained using iodine-glycerol in comparison to LPCB [2]. In iodine-glycerol method, iodine acts as fungicide as well as an agent for staining fungal structures. Glycerol prevents rapid drying of the wet preparation. In contrast to phenol, KOH and formaldehyde, iodine has no potential biohazards [2]. Furthermore, while comparing microscopic morphology of fungi, best results were achieved with LPCB and iodine glycerol. Both preparations had more transparency and high clarity. Thin delicate hyphae and semitransparent spores were visualized clearly which allowed rapid identification. However, we observed that iodine-glycerol and Congo red formaldehyde preparations were unsatisfactory for detection of fungi in clinical samples. The sensitivity of iodine-glycerol and Congo red formaldehyde methods were 59.3% and 43.8% [Table/Fig-5]. This may be due to the inability of these two methods to visualize fungal elements embedded deeply

	Sensitivity	Specificity	Positive predictive value	Negative predictive value
KOH wet preparation	65.6%	93.9%	70%	92.6%
Iodine glycerol wet preparation	59.3%	98.6%	90.4%	91.8%
Congo red formaldehyde wet preparation	43.8%	97.9%	82.3%	88.9%

[Table/Fig-5]: Comparison of three methods for microscopic detection of fungi in clinical samples

in tissue as keratin and tissue materials were not digested by a keratolytic agent. Furthermore, Congo red formaldehyde method also had limitations of rapid fading of fluorescence on light exposure and drying. As a result, Congo red formaldehyde preparations were not suitable for long term preservation.

In conclusion, Iodine-glycerol preparation was found to be a promising technique for identification fungal isolates which may be employed as a non-hazardous and safer alternative to LPCB for fungal identification.

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