

Original Research Article

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Efficacy of Iodine-Glycerol versus Lactophenol Cotton Blue for Identification of Fungal Elements in the Clinical Laboratory

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ABSTRACT

Keywords

LPCB, Iodine-Glycerol, Lugol's Iodine, Fungal Morphology, Transparency, Staining Characters.

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In the modern era, there is a lot of concern towards mycology due to an expanding array of fungal infections. Though Lacto Phenol Cotton Blue is widely used for staining, there is a necessity to develop an alternate equipotent stain which can replace it due to its high level of tumorigenic and hazardous nature. The novel stain should be eco friendly, safe with good visual clarity and staining characteristics. With this intention we have analysed a novel staining reagent Iodine-Glycerol. Various clinical samples and fungal stock cultures were analysed simultaneously using Lacto-phenol Cotton Blue and Iodine Glycerol using techniques like teasing technique, slide culture technique and adhesive tape technique. Parameters like degree of transparency, visual clarity, resolution, contrast, staining characteristics like uniformity, formation of artifacts were analysed for better demonstration of fungal morphology. Iodine-Glycerol is a better alternative to Lacto-phenol Cotton Blue for the demonstration of fungal morphology in the clinical microbiology laboratory. It is eco-friendly, non carcinogenic and much potent staining reagent. It is necessary to carry further research as there are no specific guidelines regarding the preparation of the Iodine-Glycerol staining reagent.

Introduction

Microscopic observation of wet mounts remains the most widely used method in clinical microbiology laboratories for identifying filamentous fungi based on their characteristic morphological features. The standard technique for the microscopic examination of fungal cultures is the lactophenol cotton blue (LPCB) slide tease method or the adhesive tape method. It ascertains the significance of an isolate which may otherwise be presumed as a mere insignificant artifact.

However, the phenol component of lactophenol cotton blue is carcinogenic and hence it is imperative to search an alternate staining reagent (Rodriguez-Tudela *et al.*, 1991). Lactophenol cotton blue is preferred universally for its usage as a fixative, staining and mounting medium. Lactophenol cotton blue is widely used to study the morphological features of fungal isolates. It is available in plain form as well as a combined form with polyvinyl alcohol. Teased preparation is the most common

method used in the laboratory. Main components of lactophenol cotton blue are phenol, lactic acid, glycerol and cotton blue. Phenol acts as a disinfectant, lactic acid preserves the morphology of fungus, glycerol acts as a hygroscopic agent which prevents drying and cotton blue stains the outer wall of fungus.

Phenol, an essential component in the LPCB stain has mutagenic and tumorigenic properties. It is highly corrosive and is toxic to aquatic life. Phenol is hazardous to laboratory personnel and the environment (IPCS *et al.*, 2016). This creates a clamour for an alternative eco-friendly, safe and equally functional fungal mount medium. Lugol's Iodine is a potent fungicide as it reacts with thiol groups in enzymes and proteins and hence can functionally replace the phenol component in Lacto phenol cotton blue. Although iodine solution in combination with chloral hydrate (Melzer's solution) is currently being used in clinical mycology laboratories, it has never been used as a mounting medium for microscopic identification of fungi. Also (Baszkowski *et al.*, 2006), chloral hydrate is known to be a hazardous substance. The Iodine component in Lugol's Iodine stains the outer wall of fungus and can functionally replace cotton blue as a staining reagent. Addition of 0.25% pure Glycerol to Lugol's Iodine can potentiate the hygroscopic nature of the Glycerol-Iodine. Hence, we examined the possibility of using iodine-glycerol as an alternative to LPCB and to evaluate its usefulness for wet mount preparations for microscopic observation and identification of certain clinical isolates of filamentous fungi.

Materials and Methods

The present study was conducted at in the months of July, August and September

2016. It is an observational study. It was conducted as a Short Term Studentship (STS Project) under Indian Council of Medical Research. Institutional Ethical committee approval was taken before conducting the study. Lactophenol Cotton Blue, Lugol's Iodine and pure Glycerol were purchased from Hi-Media. 0.25 ml of pure Glycerol was added to 99.75 ml of distilled water to prepare 0.25% Glycerol. Equal quantities of 0.25% Glycerol and Lugol's Iodine were added to prepare the final stain Glycerol-Iodine. 32 clinical samples of suspected fungal infection were processed. Specimens like corneal scrapings, bits of tissue, nail clippings, hair plucks, sputum, bronchial washings, skin scrapings, etc were received and further processed. Low density specimens like skin scrapings, corneal scrapings, sputum etc were kept in 10% potassium hydroxide for 30 minutes to dissolve the cementing substance holding the keratinised cells followed by thorough analysis under low power objective for the presence of fungal elements. High density specimens like nail clippings, bits of tissue etc were kept in 40% potassium hydroxide and left incubated overnight at 37 degrees followed by analysis for fungal elements. Simultaneously the specimen was mounted with Lactophenol Cotton Blue and Glycerol Iodine stain. For superficial fungal infections, adhesive tape technique is used to obtain material from clinical site of infection. Alternatively 28 filamentous fungi from stock cultures were also tested using teased preparation, slide culture and adhesive tape techniques. Various criteria like the degree of transparency, staining characteristics, visual clarity and better demonstration of the morphology of the fungus under study are taken as measuring points to compare the efficacy of Iodine Glycerol with Lacto Phenol Cotton Blue stain.

Limitations: As the study is subjective, intra-observer bias might have taken place.

Preparation of slide mounts using teasing technique

Requirements

- 1) Clean glass slides
- 2) Cover slips
- 3) Teasing needles
- 4) Staining Reagents (LPCB, Glycerol Iodine)
- 5) Bunsen flame
- 6) Sabouraud's Dextrose Agar slant with adequate fungal growth
- 7) Straight wire bent to form a spud
- 8) Microscope (Reporting)

Procedure

- 1) A clean glass slide is taken and a drop of the required staining reagent is placed at the centre of the slide.
- 2) Inoculating straight wire with spud is sterilised by holding in Bunsen flame, just above the blue cone till the entire wire becomes red hot.
- 3) After a waiting period of 15 seconds, a small amount of fungal growth is removed from the sabouraud's dextrose agar using the inoculating wire with spud.
- 4) Adequate sterilisation of the upper end of test tube containing SDA with fungal growth is maintained by flaming.
- 5) The fungal growth is transferred on to the stain on the slide, followed by gentle teasing (separation of fungal structures) using the appropriate teasing needles.
- 6) If necessary, a drop of stain is added gently to prevent drying.
- 7) The coverslip is held between thumb and index finger and one edge of the drop of

stain is touched as it spreads along one edge of the coverslip

- 8) The coverslip is gently lowered onto the slide to avoid air bubbles, followed by observation under low power and high power objective of the microscope.
- 9) Inoculating wire and teasing needles are sterilised for future use.

Preparation of slide mounts using Slide Culture technique

Requirements

- 1) Clean glass slides
- 2) Bent glass rod
- 3) Petri dish
- 4) Corn Meal agar
- 5) SDA with fungal growth
- 6) Slide, Cover slips, moist cotton
- 7) Staining reagents, Bunsen flame, spud

Procedure

- 1) Sterile microscopic slide is kept on a bent glass rod at the bottom of a petri dish
- 2) One square centimetre block of cornmeal agar is kept on the slide
- 3) Fungal strain under identification is kept at the four sides of agar block, covered with a sterile coverslip and incubated at 25 C in a BOD incubator
- 4) Moist cotton piece (with distilled water) is kept on filter paper to avoid drying
- 5) The block is monitored daily for the growth
- 6) Once growth appeared, the coverslip from block with growth is transferred on the staining reagent on a clean slide and observed under microscope.

Preparation of slide mounts using adhesive tape technique

Requirements

- 1) Adhesive tape
- 2) Clean slide, coverslips
- 3) Staining reagents
- 4) Potassium hydroxide

Procedure

- 1) Scotch tape is applied to the clinical site of superficial skin infection or fungal culture and then transferred to a clean slide
- 2) A drop of Potassium hydroxide is placed between tape and glass slide so that the cementing material is dissolved

- 3) After waiting for some time, appropriate staining reagent is added to study the fungal morphology

Results and Discussion

It was observed that the visual clarity and the degree of transparency were more with Iodine-Glycerol as compared to Lactophenol Cotton Blue. Contrast is reasonably good with LPCB but it is much satisfactory with Iodine-Glycerol. Better resolution is observed with Iodine-Glycerol. The staining characteristics like uniformity, clarity of the various morphological structures, lack of any artifacts due to the staining material on prolonged storage etc were better appreciated with Iodine – Glycerol than LPCB.

Table.1

Sl No	Parameter Observed	Lacto-Phenol Cotton Blue	Iodine-Glycerol
1	Degree of Transparency	Less	More
2	Visual Clarity	Less	More
3	Contrast	Good	Satisfactory
4	Resolution	Good	Excellent
5	Staining Characteristics	Good	Excellent
6	Demonstration of Morphology	Good	Excellent
7	Uniform staining	Good	Better than LPCB
8	Artifacts on prolonged usage	More	Less

Fig.1 Lactophenol Cotton Blue and Iodine-Glycerol stains



Fig.2 LPCB of *Rhizopus*

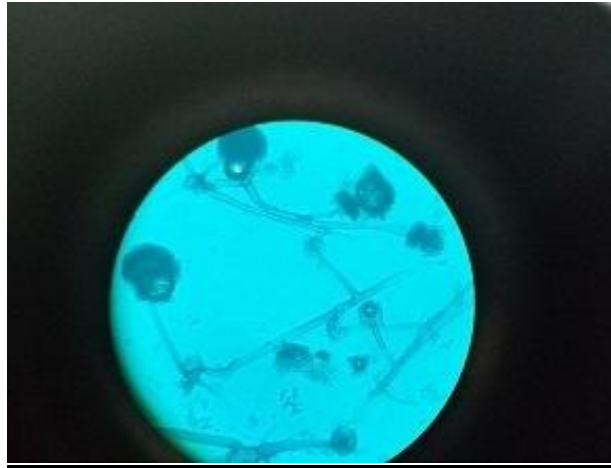


Fig.3 Iodine-Glycerol mount of *Microsporium gypseum*



In the present study, it was found out that more light is able to pass through the Iodine-Glycerol stain than Lacto-phenol Cotton Blue making it more transparent. This could be attributed to the intensity of the staining reagent. Lugol's Iodine is comparatively less intense stain than Lacto-phenol Cotton Blue.

Also the process of mixing Lugol's Iodine with 0.25% Glycerol in equal proportion has considerably decreased the colour intensity. Thus it can be inferred that lighter stains permits better light penetration than the dark stains.

As visual clarity and power of resolution are directly proportional to the numerical aperture, which further depends on the amount of light penetrating through the staining material, it can be said that Lugol's Iodine has better visual clarity and power of resolution than Lacto-Phenol Cotton Blue. Contrast is much better in case of Iodine-Glycerol than Lacto-phenol Cotton Blue. This is because the outline of fungal structures appear dark against the background of staining reagent. Also minute structures like conidia are better stained under Iodine-Glycerol than Lacto-phenol

Cotton Blue. We have observed that on prolonged storage, artifacts are more pronounced with LPCB than Iodine-Glycerol leading to lack of uniformity of the preparation. The observations in my study correlated with that of Vignesh *et al.*, (2008) and Vacharavel shamly *et al.*, (2014). We could not get adequate references due to dearth of information on the topic.

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Suggestions

The present study strongly emphasise replacement of lactophenol cotton blue with iodine-glycerol as a better alternative for the demonstration of fungal morphology in the clinical microbiology laboratory. However there is a dearth of information and clear protocol regarding the preparation of Iodine-Glycerol stain. The present study made an endeavour mixing equal quantities of 0.25% Glycerol and Lugol's Iodine. The present study suggested future research altering the concentration of glycerol and the proportions of mixing glycerol and lugol's iodine for a better outcome in terms of the

degree of transparency, staining characteristics, visual clarity and demonstration of fungal morphology in the clinical microbiology laboratory

References

- Baszkowski, J., Renker, C., Buscot, F. 2006. *Glomus drummondii* and *G. walkeri*, two new species of arbuscular mycorrhizal fungi (Glomeromycota). *Mycol. Res.*, 110: 555-66.
- IPCS. 1994. Phenol: Health and Safety Guide. Geneva: WHO. Available from: http://www.inchem.org/documents/hsg/hsg/hsg88_e.htm. [Last Accessed 2012 Jun 16]
- Rodriguez-Tudela, J.L., Aviles, P. 1991. Improved adhesive method for microscopic examination of fungi in culture. *J. Clin. Microbiol.*, 29: 2604-5.
- Vacharavel Shamly, Arunava kali, Sreenivasan Srirangaraj, Sivaraman Umadevi *et al.*, 2014. *J. Clin. Diagn. Res.*, 8(7): DL01-DL02.
- Vignesh, R., Sekar, R., Shankar, E.M., Kumarasamy, N., Murugavel, K.G., Irene, P., *et al.* 2008. Wet mounting using iodine-glycerol provides a semi-permanent preparation for microscopic observation of faecal parasites. *J. Med. Microbiol.*, 57: 679-80.

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