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Masked comparison of trypan blue stain and potassium hydroxide with calcofluor white stain in the microscopic examination of corneal scrapings for the diagnosis of microbial keratitis

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Purpose: To evaluate the efficacy of trypan blue in direct microscopic examination of corneal scrapings in the diagnosis of non-viral microbial keratitis. **Methods:** In a prospective, interventional, masked study, 82 consecutive patients were investigated. Direct microscopic examination of the corneal scrapings involved three smears stained with potassium hydroxide with calcofluor white (KOH + CFW), Gram stain (not analyzed), and trypan blue stain and culture for bacteria, fungus, and *Acanthamoeba*. While KOH + CFW stained slides were examined under a fluorescence microscope, trypan blue-stained slides were examined by two microbiologists (masked to KOH + CFW and culture results) under normal light microscopy. Thirty samples were reexamined for interobserver and intraobserver variability. **Results:** Out of 82 samples, fungal/fungus-like elements were seen in 48 (58.5%) in KOH + CFW and 38 (46.3%) in trypan blue stain. One microsporidial case, detected in KOH + CFW was negative in trypan blue stain (culture not done). Fungal growth was positive in 23 out of 81 (28.3%) cases cultured, single bacterial species in 18 (22.2%), *Pythium insidiosum* in three, mixed bacteria and fungus/*Pythium* in 7 (8.6%), *Acanthamoeba* in none and 30 (37.0%) samples were sterile. With culture as gold standard, the respective sensitivity (84.9%, 75.7%) and specificity (90.9%, 68.2%) of KOH + CFW stain and trypan blue stain were comparable (p = 0.16, *P* = 0.06). The interobserver linear weighted kappa score between the two microbiologists was 1.00 while it was 0.86 for intraobserver agreement. **Conclusion:** Trypan blue stain, an easily available dye to ophthalmologists, is highly efficacious in the diagnosis of fungal keratitis.

Key words: Calcofluor white, corneal scraping, microbial keratitis, microscopic diagnosis, potassium hydroxide, trypan blue

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Infectious keratitis is a serious blinding disease if not treated promptly with appropriate therapy. Prompt treatment is possible with quick identification of microorganisms and initiation of appropriate therapy. Direct examination of corneal scraping smears using 10% potassium hydroxide (KOH) with or without calcofluor white (CFW) stain, Grams stain, and culture are part of the standard protocol for the laboratory diagnosis of non-viral keratitis.[1] Sharma *et al*.[2] had shown that 10% KOH was comparable to KOH + CFW in terms of specificity and predictive values with no statistically significant difference although the sensitivity of the latter was higher. However, the use of KOH + CFW requires a fluorescence microscope. Use of 10% KOH remains to date a simple and quick direct smear examination of corneal scrapings in the identification of fungus, especially in regions where fungal keratitis is more common.[3]

The main disadvantage of 10% KOH mount is that the visibility of the fungus is hindered due to the transparent nature of the preparation. The filaments to the untrained eye can be missed out altogether, especially if the filaments are scanty or broken. The recognition of other pathogens such as *Acanthamoeba* cysts and microsporidial spores is even more challenging as these entities can be easily missed or confused with other cellular or acellular debris on the mount. Ranging from low to high values, sensitivity, and specificity of this procedure have been extensively published in the literature.[2,3] The reports suggest the need for considerable experience and skill. Efforts have been made in the past to add color to 10% KOH to improve visibility, one notable dye being lactophenol cotton blue.[4] Other dyes that have been used are simple stains such as aniline blue and methylene blue.[5]

Compared to 10% KOH, higher sensitivity of 0.1% CFW stain along with 10% KOH (KOH + CFW) has been published before and this procedure is much preferred in laboratories equipped with a fluorescence microscope. The sensitivity and specificity of KOH + CFW stain in the diagnosis of fungal keratitis have been reported to be 85% and 89%, respectively.[1] However, not all laboratories and ophthalmology practice set-ups can invest in a fluorescence microscope. Consequently, only 10% KOH is in wide use including secondary eye care centers in rural India.[4,6] We recently reported our experience with 10% KOH in secondary centers where a single ophthalmologist is employed and resources are limited.[6] All such centers are provided with a binocular light microscope and facility to perform corneal scraping examination using 10% KOH and Gram stain for the diagnosis of microbial keratitis.

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In order to improve upon the results, we decided to use trypan blue (for staining) which is available in the secondary centers as this dye is readily available and routinely used for performing capsulorhexis in cataract surgery. The leftover dye from the operating rooms is available to ophthalmologists at no cost. However, prior to implementing the procedure, we wanted to evaluate the efficacy of the procedure in comparison to the well-established methods of KOH + CFW and the gold standard of culture. This study investigated consecutive cases of microbial keratitis for this purpose. In a masked study, the microscopy results of the corneal scrapings examined by KOH + CFW were compared with trypan blue stain for sensitivity and specificity. Two microbiologists (SM, SS) with 3 and 30 years' experience respectively in diagnostic ocular microbiology reexamined 30 samples for interobserver and intraobserver variability in a masked manner with the slides renumbered by a technician (BY) in the laboratory.

Methods

Collection of corneal samples

During September and October 2020, a total of 82 consecutive patients, clinically diagnosed as non-viral microbial keratitis, were included in the study. All patients underwent complete slit-lamp biomicroscopy of the cornea and anterior segment followed by corneal scraping under topical anesthesia. Corneal scrapings were collected under slit lamp magnification using surgical blade #15 on Bard-Parker handle and provided on three sterile glass slides. While the tissue material was spread over a small (approximately 1 cm diameter) circle (marked on the back of the slide with glass marker) on two slides, the third sample was just placed within the circle. These scrapings were not collected in any order, however, were always collected before inoculating culture media. Corneal scrapings were processed as described by us earlier.[7]

Processing of the slides for direct microscopy

The slide with corneal scraping placed in the center was used for fluorescence staining using one drop of 10% KOH and one drop of 0.1% CFW containing Evan's blue, as described earlier.[7] A coverslip was placed on the smear and the slide was examined under a fluorescence microscope with 395 nm UV filter (Olympus, BX51). Of the two slides with thinly spread smear, one was used for Gram stain (HiMedia, Mumbai, India) and the other for trypan blue stain (Trypan blue solution, aqueous solution 0.06%, Contacare Ophthalmics and Diagnostics, Vadodara, Gujarat, India). To 5 ml of trypan blue pooled (using 2 ml syringe aseptically from several vials with leftover solution from the operating room) into a sterile eyedropper bottle [Video 1], 0.5 mL of glycerol was added (0.025%). One drop of this solution was placed on the smear, mounted with a coverslip [Video 1], and examined under 10×, 20×, and 40× objective lenses of the microscope using normal halogen bulb white light.

Masking and repeatability testing procedure

The KOH + CFW stained smear was seen by any of the two microbiologists (SS, SM) and the results were recorded such that they were not available to the two microbiologists who examined the trypan blue-stained slides. The identity of the patient number for each sample was concealed for the trypan blue-stained slides by having a laboratory technician (YB) number the slides consecutively and providing them to the microbiologists for observation and recording. The culture findings were also not available to the microbiologists who examined the trypan blue-stained slides. The findings of trypan blue stained slides were recorded separately and compiled later by one of the authors (VMR) for statistical analysis. The same person also collected the results of KOH + CFW and culture data.

For testing repeatability of trypan blue stain, 30 smears were consecutively examined for the second time by one of the microbiologists (SM) blinded to the earlier results by way of renumbering of the slides by a technician (BY). These slides were also examined by the second microbiologist (SS) who was masked to the results of the other microbiologist, for calculation of inter-observer variability.

Statistical analysis

The statistical analysis was performed using the software STATA v14.2 (StataCorp, College Station, TX, USA). Categorical data were described in proportions. Data of KOH + CFW staining and trypan blue staining were recorded as true positive, false positive, true negative, or false-negative with respect to culture as the gold standard. Sensitivity, specificity, positive predictive value, and negative predictive value were estimated after cross-classification. When culture was taken as a gold standard, pure bacterial cultures were removed from the analysis. The sensitivity and specificity of KOH + CFW and trypan blue were compared by the Stuart-Maxwell test for marginal homogeneity. A P value of <0.05 was considered statistically significant. With KOH + CFW as gold standard, all samples were analyzed to estimate the diagnostic performance of trypan blue staining. Linear-weighted kappa scores were estimated to assess the intra-observer and inter-observer concordances. A value of 0.75 or more was considered a measure of excellent concordance. The proportions of grading between two techniques were compared by McNemar test; here, a P -value of <0.0125 was considered statistically significant after Bonferroni correction for multiple testing.

Results

In total, 82 corneal scrapings from 82 consecutive patients with clinical diagnosis of microbial keratitis were analyzed. Fungal/fungus-like filaments were graded by semi-quantification based on the number of filaments, viz., grade zero—none, grade one = 1–5, grade two = 6–10, grade three = 10–plenty. While bacteria were not expected to be seen owing to the maximum magnification limit of x400, one corneal scraping was positive for microsporidial spores and none showed presence of *Acanthamoeba* cysts.

Of the 82 samples, direct microscopy showed presence of microorganisms in 48 (58.5%) on KOH + CFW stain (Fungal/fungus-like elements-47, Microsporidia-1) and presence of fungal/fungus-like elements in 38 (46.3%) on trypan blue stain. The sample positive for microsporidia was not cultured. Fungal growth was noted in 23 out of 81 samples cultured (28.3%), while 18 (22.2%) showed bacterial growth of single species and *Pythium insidiosum*, an oomycete (morphologically fungus-like) was cultured in three cases. Culture yielded no growth in 30 (37.0%) samples. Mixed growth of bacteria and fungus was seen in 7 (8.6%) cases, however, there were no samples positive for *Acanthamoeba.* Organism-wise distribution of KOH + CFW and trypan blue stain positivity in corneal scrapings is given in Table 1.

Direct microscopy showed presence of fungal elements in 90.9% on KOH + CFW stain and 84.9% on trypan blue stain. Table 2 shows the sensitivity, specificity, positive predictive value, and negative predictive value of KOH + CFW stain and trypan blue stain when compared to culture as a gold standard. Both the respective sensitivity (84.9% vs 75.7%) and

Table 1: Organism-wise distribution of KOH+CFW and trypan blue stains positivity of corneal scrapings

Organism group	Organisms with distribution	KOH+CFW Positive	Trypan blue positive
	n=82	*n*=48	*n*=38
Fusarium spp.	15	15	14
Aspergillus spp.	5	4	4
Dematiaceous fungi	2	1	1
Unidentified dematiaceous fungus	1	1	1
Pythium insidiosum	3	3	3
Single bacterial species	18	3	1
Mixed bacteria and fungus	7	6	5
Microsporidia	1	1	0
Culture negative	30	14	9

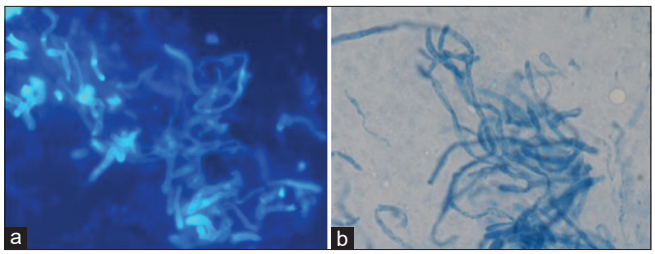

Figure 1: Corneal scraping showing (a) broad, aseptate fungus-like filaments with ribbon-like folds suggestive of *Pythium* species (KOH + CFW stain, x400). Similar filaments are seen in trypan blue stained smear of corneal scraping (b, x400)

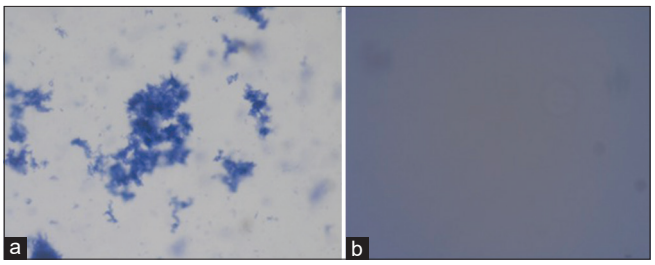

Figure 2: Dropped on a slide, trypan blue aggregates in blue clumps leaving a colorless background when mixed with one drop of 10% potassium hydroxide (a, magnification x100). Mixed with glycerol, it forms a smooth blue emulsion (b)

Table 2: Sensitivity and specificity of KOH+CFW and trypan blue against culture as gold standard

	KOH+CFW stain	Trypan blue stain
Sensitivity	90.9% (CI: 74.5-97.6)	84.9% (CI: 67.3-94.3)
Specificity	51.7% (CI: 32.9-70.1)	69.0% (CI: 49.1-84.0)
Positive predictive value	68.2% (CI: 52.3-80.9)	75.7% (CI: 58.5-87.6)
Negative predictive value	83.3% (CI: 57.7-95.6)	80.0% (CI: 58.7-92.4)

CI: Confidence interval

Table 3: Trypan blue stain compared with KOH+CFW stain taken as gold standard

Statistics	Value %	95% CI
Sensitivity	77.1	62.3-87.5
Specificity	97.1	83.0-99.9
Positive predictive value	97.4	84.6-99.9
Negative predictive value	75.0	59.4-86.3

specificity (90.9% vs 68.2%) of KOH + CFW stain and trypan blue stain were comparable ($P = 0.16$ and $P = 0.06$ retrospectively).

Corneal scrapings from 30 out of 81 cases (37.0%) were culture-negative, out of which 14 (46.6%) were positive for fungal/fungus-like elements by KOH + CFW stain and 9 (30.0%) were positive by trypan blue stain. Eight out of nine trypan blue positive samples were also positive in KOH + CFW. Taking KOH + CFW as gold standard, the sensitivity of trypan blue was 77.1% and specificity was 97.1%. Table 3 shows the sensitivity and specificity of trypan blue stain when KOH-CFW stain was taken as gold standard.

An analysis of the semi-quantification of a load of fungal/fungus-like elements detected by the two methods showed that trypan blue method detected grade three filaments in significantly lesser cases (9 versus 23, $P = 0.0005$) compared to KOH + CFW, with consequent increase in grade 0 cases (43 versus 34, $P = 0.012$). The interobserver and intraobserver linear weighted kappa scores for agreement were excellent. The kappa score was 1.00 (95% confidence interval CI: 1.00–1.00) between the two microbiologists. The intraobserver agreement showed a kappa score of 0.86 (95% CI: 0.67–1.00).

Discussion

Way back in 1956, Boedijn *et al*.[8] have shown that trypan blue can be used as a stain in the identification of fungal filaments. Classified among vital stains, trypan blue is an azo dye with a negative charge which can be used in the staining of dead and live cells.[9] We did try but ultimately decided against applying these characteristics in our study to differentiate between live and viable fungal elements, owing to the realization that a dry corneal scraping is likely to abound with mostly dead filaments on a glass slide. With an intention to employ trypan blue staining for corneal scrapings in secondary rural centers (currently using 10% KOH mount) in our village vision complex program, we first wished to determine if the method would fare well in comparison to the gold standards.

The gold standard is the best single test that is considered the current preferred method of diagnosing disease and for infectious diseases caused by culturable microorganisms, it is usually the culture results. However, we chose two gold standards- one was culture and the other was KOH + CFW

staining method. Using KOH + CFW as the gold standard seemed appropriate based on our earlier report in a small number of cases wherein 10% KOH mount was statistically comparable to the fluorescent method of KOH + CFW, although more cases could be detected by the latter technique which allowed detection of very tiny fungal fragments.[2]

In this study, for the detection of fungal/fungus-like elements in corneal scrapings, the sensitivity of trypan blue preparation was 77.1% and the specificity was 97.1% with KOH + CFW as the gold standard [Table 3].

We are yet to compare the trypan blue stain with 10% KOH, however, the earlier published data suggest similar performance by both. We also analyzed our data taking culture as gold standard and were surprised to find a reversal in the level of sensitivity and specificity- high sensitivity of 90.9% and 84.9% but low specificity of 51.7% and 69.0%, respectively, for KOH + CFW and trypan blue stain respectively with low positive predictive values for both. However, the difference between the stains with respect to sensitivity and specificity was not significantly different. This finding can be attributed to fallacies of the fungal culture to serve as gold standard. Non-viable fungal filaments may fail to grow in culture and sample variation in multiple corneal scrapings may account for culture being a less than ideal gold standard.[2,10] Inability of the gold standard to be positive in presence of disease can lead to false positives that will affect the specificity of a test that indicates higher than real false positives.[11] In this study, fungal/fungus-like filaments were seen in 14 (KOH + CFW) and 9 (trypan blue) out of 30 culture-negative samples. Presence of organisms such as filamentous fungi, yeast, microsporidia, and *Acanthamoeba* cysts that can be discerned well in KOH + CFW should be taken as indicative of disease irrespective of growth in culture. As mentioned earlier, sample variation can well account for lack of growth and microsporidia is unculturable in ordinary media. The same holds true for *Pythium* sp., an oomycete, that microscopically resembles fungus and clinically mimics fungal keratitis.[12] In this study, there were seven patients who were diagnosed with *Pythium insidiosum* keratitis, all of which were positive in direct microscopy of the corneal scraping by both KOH + CFW and trypan blue methods [Fig. 1a and b] showing typical broad, aseptate fungus-like filaments with ribbon-like folds. However, the organism grew in culture in three cases in pure culture, two cases in mixed culture with bacteria (*Staphylococcus saprophyticus*, *Streptococcus mitis*) and failed to grow in two cases.

The technique of using trypan blue for staining and observation of corneal scrapings has been described in detail in this communication. The addition of glycerol in trypan blue kept the smear moist for a long time (tested up to 3 days). It may be noted that potassium hydroxide (KOH) was not added to trypan blue as it was found to produce aggregates of dark blue clumps [Fig. 2a] interfering with the reading of the smears. Trypan blue remained a smooth emulsion on the addition of glycerol [Fig. 2b]. Based on this experience of clumping with KOH, we believe that the single case recently described by Prasher *et al*.[13] would have faced the same issue which has not been mentioned by the authors. In fact, Fig. 1b of their article shows the aggregates. The technique described by them involved drying the smear in a drop of KOH which is bound to form KOH crystals and unnecessary loss of time. In our experience, the smears can be examined immediately after placing a drop of trypan blue containing glycerol and a coverslip. Scraping from corneal ulcer with necrosed tissue may not require digestion with KOH and in any case, a wet smear can always be pressed on the coverslip to flatten the smear if too thick. We also recommend that the corneal scraping be thinly spread over the slide.

Trypan blue stain in this study without KOH was well comparable to KOH + CFW in terms of all parameters that are normally used for comparison of diagnostic tests.[11] For logical conclusion, it may be worthwhile to conduct a prospective study to directly compare the efficacy of 10% KOH method with 0.06% trypan blue, nevertheless, results of this study are encouraging enough to launch the use of this technique in ophthalmology setups where ophthalmologists themselves are engaged in determining the microbiological diagnosis of patients with corneal ulcers in their clinic.

Conclusion

This was a prospective masked comparative study to evaluate the efficacy of trypan blue stain in the diagnosis of microbial keratitis. With high sensitivity and specificity along with good interobserver and intraobserver scores, the stain was efficacious and comparable to potassium hydroxide with calcofluor white stain in the direct microscopic diagnosis of fungal keratitis.

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Conflicts of interest

There are no conflicts of interest.

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