COMPARISON OF DIFFERENT STAINING TECHNIQUES AND CULTURE MEDIA USED FOR DIAGNOSIS OF INFECTIVE KERATITIS

MALIK M.,[1] JAVED I.,[2] MUSHTAQ S.,[3] ANWAR M. S.[4] AND AKHTAR F. K.[5]
Department of Pathology, [1-3]Ameer-ud-Din Medical College/Post Graduate Medical Institute
[4]Combined Military Hospital, (National University of Medical Sciences)
Department of [5]Ophthalmology, Medical officer at Lahore General Hospital, Lahore – Pakistan

ABSTRACT

Background and Objectives: Corneal scraping is ideal diagnostic procedure for infective keratitis. It is needed to find simple and proper microbiological investigative modalities for corneal scrapings by using direct microscopic methods, which are easily available and worthy in detection of micro-organisms so that eye clinics develop appropriate and well-timed diagnosis, treatment for averting visual loss. The study was designed to evaluate different staining techniques and culture media used for diagnosis of infective keratitis. It is a descriptive study carried out at Lahore General Hospital, Lahore during June, 2016 – June 2017, and the samples were processed according to SOPs at Microbiological laboratory of PGMI.

Methods: Corneal scrapings were collected from fifty patients who were clinically diagnosed as infective keratitis by Ophthalmologist on clinical examination. The corneal scrapings were immediately inoculated on Chocolate agar, Blood agar, MacConkey agar and Sabouraud's Dextrose agar with antibiotics. The scrapings were stained with Gram's staining technique, Kinyoun staining, Giemsa stain and KOH wet mount preparation. Identification of bacterial and fungal pathogens was done by Microbiological SOPs in laboratory.

Results: Out of 50 cases of corneal scrapings, 9 (18%) cases of bacterial pathogen were confirmed on Grams staining. One (2%) case of Nocardia spp. was confirmed on Kinyoun staining. 17 (34%) cases of fungal pathogens were confirmed on KOH/LPCB wet mount. Gram staining was 87.88% sensitive and 94.12% specific for diagnosing both bacterial and fungal pathogens. KOH/LPCB were 76.19% sensitive and 96.55% specific for only identification of fungal pathogens. 33 (66%) cases were confirmed in laboratory on cultures.

Conclusion: Use of direct smear microscopy of corneal scrapings should be endorsed by different techniques and cultures from the patients who are clinically diagnosed as infective keratitis. Although culture is considered the gold standard but direct microscopy of smears provides prompt information of causative microorganisms.

Keywords: Infective keratitis, Corneal scrapings, Direct microscopy, Culture, Staining techniques.

INTRODUCTION

Infective keratitis is a leading cause of ocular morbidity and the commonest cause of unilateral blindness worldwide especially in low-socioeconomic settings. The incidence of infective keratitis in these settings is estimated up to 800 per 100,000/year, which is 70 times higher as compared to high-socioeconomic settings.[1]

The spectrum of keratitis is diverse in association with pathogens and it includes bacteria, viruses, fungi and protozoa.[2] When there is any suspicion of an infective keratitis, it should be thoroughly investigated. This will be helpful in making proper diagnosis and guide the clinician in therapeutic approach.[3]

For appropriate treatment microbiological diagnosis and clinical expertise is required. A huge range of conventional methods and molecular based techniques are present that provide information related to unknown organisms that are associated with corneal infections.[4] Corneal scraping examination is one of the ideal diagnostic procedure for infective keratitis.[5] These scrapings are used for direct microscopic methods which are easily available and worthy in detection of microorganisms.[6] Although culture is also used for corneal scrapings and considered as a gold standard for identification of pathogenic organism of infective keratitis[7] but direct microscopy of smears provides prompt information of causative microorganisms.[6]

48 Corresponding Author: Dr. Muna Malik, MBBS, Demonstrator Pathology
Ameer-ud-Din Medical College/Post Graduate Medical Institute, Lahore – Pakistan
Cell: 03214260644 E-mail: mmkamboh@hotmail.com

These staining techniques are beneficial as they are easy to do and have high sensitivity and specificity.[6] For rapid identification of fungal pathogens, conventional techniques include 10% KOH and lacto phenol cotton blue wet mounts.[8] Gram and Giemsa staining techniques are used for identification of bacteria[6] and Modified Ziehl-Neelsen staining for Nocardia identification.[9] These staining techniques provide a preliminary diagnosis, when culture results are pending.[10]

The present study was designed to identify bacterial and fungal pathogens in corneal scrapings of infective keratitis, by comparing different staining techniques and culture media inoculation. The corneal scraping is considered a precious sample and usually remained undiagnosed because of its small quantity and presence of fastidious microorganisms. Corneal scrapings direct wet mount preparations and staining techniques provide a preliminary diagnosis before culture yield and would be helpful to start empirical treatment of the patient, thereby improving visual prognosis.

METHODOLOGY

Inclusion Criteria

1. Clinically suspected cases of Infective keratitis.
2. Corneal scrapings collected before start of antimicrobial or antifungal drugs.

Exclusion Criteria

1. Patients who were on antimicrobial or antifungal drugs.

Corneal scrapings were collected from the patients by ophthalmologist in operation theater of Eye Department, Lahore General Hospital, Lahore. In this study, fifty patients were included. The patient's name, age, sex, date of collection, brief clinical history including onset, duration, history of trauma/corneal foreign body, and any other corneal issues were taken and recorded in proforma I. All further microbiological processing of the scrapings were recorded in proforma II.

Topical anesthetic, Proparacaine was instilled in the eye. The necrotic tissue and loose mucus was removed from the surface of the ulcer. The margins and base of the ulcer was scraped by disposable scalpel blade no 15 by the ophthalmologist.[11]

The corneal scrapings taken by the ophthalmologist was immediately inoculated on Blood, Chocolate, MacConkey and Sabouraud's dextrose agar with the help of sterilized wire loop in the operation theater. After inoculating the scrapings on the culture agar plates, the scrapings from cornea were smeared on glass slides with the help of sterilized wire loop. The smears were air dried and fixed by heating for Gram's staining and Kinyoun staining and with alcohol for Giemsa stain. The slides were labeled, with serial numbers, name of the patient and placed in a slide transport box.

These slides were taken to the laboratory and were processed for Wet mount preparation for rapid diagnosis of any fungal pathogen in corneal scrapings. The Gram's staining was used to identify Gram positive and Gram-negative bacteria. The Gram's staining was also used for fungal detection. Fungal hyphae, spores and yeast cells if present were stained Gram positive and appeared violet in color. Giemsa staining was used to see the inclusion bodies of *Chlamydia trachomatis* in infected epithelial cells. Inflammatory cells were differentiated into mononuclear and polymerphs cells based on differential staining and nuclear characteristics. Kinyoun staining was used for *Nocardia spp*. It appeared as weak acid fast branching filaments. They also stained as Gram-positive branching filaments.

After 24 hours of incubation of inoculated culture plate, preliminary identification of bacterial pathogens was done on basis of colonial morphology like size, shape, color, surface, elevation, pigment production, presence or absence of hemolysis on blood agar of bacteria isolated on culture plates. This was followed by Gram's staining, catalase, coagulase and oxidase tests. API did the final identification upto species level.
Fungal isolates were identified by:

Growth rate was observed as rapid, moderate or slow. This was done by measuring the colonial diameter at the end of every week for 3 weeks.

Pigmentation on the surface of aerial hyphae and on reverse of colony and noting any diffusible pigment in the medium

Surface texture of colonies grown on agar plates was observed. This includes the surface whether it was glabrous or waxy, powdery, velvety, granular, downy or fluffy.

Topography of colony was noted as flat, raised or heaped.

Microscopic examination was done by making Lacto phenol cotton blue tape preparation of positive cultures.[12-14]

RESULTS

The table 1 shows that, out of 50 specimens of corneal scrapings of clinically diagnosed infective keratitis. 27 (54%) cases were confirmed in laboratory by different staining techniques. 9 (18%) cases of bacterial pathogen were confirmed on Grams staining. 1 (2%) case of *Nocardia spp*. was confirmed on Kinyoun staining. 17 (34%) cases of fungal pathogens were confirmed on KOH/LPCB wet mount preparation and no case of *Chlamydia spp*. was confirmed on Giemsa staining.

Table 2 shows comparison of sensitivity and specificity of different staining techniques used in the present study for laboratory confirmation of bacterial and fungal pathogens in corneal scrapings. Gram staining was 87.88% sensitive and 94.12% specific for diagnos-

ing both bacterial and fungal pathogens. KOH/ LPCB were 76.19% sensitive and 96.55% specific for identification of fungal pathogens. Kinyoun stain was 100% sensitive and specific for *Nocardia spp*. only. In addition, Giemsa stain was 57.14% sensitive and 96.55% specific for identification of fungal pathogens in this study.

Table 3 shows culture results of corneal scrapings of clinically diagnosed cases of infective keratitis. Out of 50 cases 11 (22%) were shown growth of only bacterial pathogens on culture. 19 (38%) cases were shown growth of only Fungal pathogens and 3 (6%) were shown growth of mixed bacterial and/or fungal pathogens. 33 (66%) cases were confirmed in laboratory on cultures.

Table 4 shows the results of laboratory findings of 50 corneal scrapings of infective keratitis patients. Out of these 50 specimens, 34 (68%) cases of infective keratitis were detected in laboratory. Only one (2%) was confirmed by microscopic techniques, 4 (8%) were confirmed on cultures and not picked by microscopic techniques and 29 (58%) cases were confirmed by both staining techniques and on culture.

Table 1: *Comparison of different staining techniques used for diagnosing Infective keratitis in microbiology laboratory.*

Microscopy Techniques	*Positive for microscopy*	
	No of cases	*% age*
Gram staining for Bacteria	09	18%
Kinyoun staining for Nocardia	01*	2.0%
Giemsa staining for Chlamydia	00	0.0%
KOH/LPCB for Fungus	17	34%
Total Positive	27	54%

*Also seen on Gram Staining.

Table 2: *Comparison of different microscopic staining techniques for laboratory identification of both Bacterial and Fungal pathogen in corneal scrapings of clinically suspected cases of Infective keratitis (n=50).*

Microscopic Staining Techniques	*No of Cases of Both Bacterial and Fungal Pathogens Identified*	*Sensitivity of Stain for Identification*	*Specificity of Stain Identification*
Gram stain	30	87.88%	94.12%
KOH/LPCB	17	76.19%	96.55%
Kinyoun stain	1	100.00%	100.00%
Giemsa stain	13	57.14%	96.55%

Table 3: *Yield of bacterial and fungal pathogens on different culture from corneal scrapings (n = 50).*

Category	*Positive for Culture*	
	No of Cases	*% age*
Growth of Bacterial pathogens only	11	22%
Growth of Fungal pathogens only	19	38%
Mixed growth of Bacteria and/or Fungal pathogens	03* **	6%
Total culture positive Cases	33	66%

*Two cases were positive for mixed growth of Bacterial and Fungal pathogens on culture.
**One case was positive for mixed growth of Bacterial pathogens on culture.

Table 4: *Comparison of culture positivity with microscopy results (n = 50).*

Category of Cases	*No. of Cases*	*% age*
Positive on Microscopy only	1*	2%
Positive on Cultures only	4	8%
Positive on both Microscopy and Cultures	29	58%
Total detected cases	34**	68%

*One case was picked on microscopy and not confirmed on culture.
**Total 34 cases were detected in laboratory, out of which 33 were confirmed on culture and one case was not confirmed on culture and detected on microscopy only.

Table 5 shows Sensitivity and specificity pattern between direct microscopic staining techniques and culture for corneal scrapings in laboratory confirmat-

ion of clinically diagnosed cases of infective keratitis. The results of present study showed 87.88% sensitivity, 94.12% specificity, 96.67% PPV and 80.00% NPV. Overall, diagnostic accuracy of the tests was 90%.

The table shows that how many cases of corneal scrapings of clinically diagnosed patients of infective keratitis were confirmed in laboratory. Out of 50 clinically diagnosed cases, 29 were true positive i.e. they were confirmed on both microscopy and culture. Sixteen cases were true negative i.e. they were negative on both microscopy and culture. One case was false positive i.e. it was confirmed on microscopy but culture was negative. Four cases were false negative they were positive on culture but negative on microscopy.

Table 5: *Sensitivity and specificity patterns between direct microscopy techniques and culture for corneal scrapings of clinically diagnosed cases of Infective Keratitis (n = 50).*

		Results of Cultures		*Total*
		Positive	*Negative*	
Results of Microscopy	Positive	29	1	30
	Negative	4	16	20
Total		33	17	50

SN = sensitivity: 87.88% (95% CI = 71.80% to 96.60%)
SP = Specificity: 94.12% (95% CI = 71.31% to 99.85%)
PPV = Positive predictive value: 96.67%
NPV = Negative predictive value: 80.00%
DA = Diagnostic accuracy: 90.00%

DISCUSSION

Corneal scrapings direct microscopic examination provides quick diagnosis and play an important role is starting the initial therapy that may be modified according to culture report later on. Thus, smear diagnosis is important for achieving optimum diagnosis and treatment. Similarly, many researches have used Gram and Giemsa staining in diagnosing bacterial pathogens in corneal scrapings and Kinyoun staining for diagnosing *Nocardia spp*. in eye infections. Many studies conducted on fungal keratitis in different countries like India used KOH wet mount for preliminary identification of fungal hyphae in corneal scrapings.[15-17]

Feilmeier *et al* from Nepal (2010) revealed importance of KOH wet mount preparation in their study by comparing performance of KOH wet mount preparation with fungal cultures and reported its sensitivity 84.85% and 80.5% respectively, which is similar with our study.[18] In this study, no *Chlamydia*l infection was identified on Giemsa staining technique. However, Giemsa staining was helpful in identifying fungal hyphae in fungal keratitis. The sensitivity and specificity of Giemsa staining reported by Boggild *et al* (2009) were similar to this study.[19] Correspondingly, Sharma *et al* (2002) documented sensitivity and specificity for smear examination as 87.1% and 83.7% and Bharathi *et al* 2006 reported as 96.1% and 98.9% respectively, which is in accordance to current study.[6]

Kumar *et al* (2011) isolated 26.5% bacterial growths, 22.5% fungal growths and 6% mixed microbial growths on culture Medias in their study, which is consistent with this study. Similarly, many researchers concluded in their studies that conventional method required several days to week for growth on culture media; newer diagnostic modalities like confocal microscopy, genetic finger printing or PCR would be helpful for early detection of bacterial and fungal pathogens in corneal scrapings.[21] However, present study was limited by these newer modalities.

It is ***concluded*** that clinical examination by the ophthalmologists is not enough for ultimate diagnosis of keratitis therefore corneal scraping are mandatory and recommended for microbiological analysis of its etiology, which will be sight saving and they provide vision to identify the causative pathogens. Based on the findings of the present study, the use of direct smear microscopy of corneal scrapings should be endorsed by different staining techniques and cultures media. The LPCB/KOH wet mount was useful for fungal hyphae detection. Gram staining would be helpful for identification of Bacterial and fungal hyphae and yeast cells, Giemsa staining for Chlamydia but it also identified fungal hyphae. Kinyoun staining was supportive in diagnosing Nocardia.

Authors' Contribution

MM: Sample collection, Data analysis and paper writing. IJ: Concept, Supervision, Literature review. SM: Supervision, Literature review. MSA: Literature review. FKA: Data analysis.

Conflict of Interest

No conflict of interest.

ACKNOWLEDGEMENT

This research was supported by PGMI, Lahore. Staff of Microbiology Laboratory of PGMI and Ophthalmology Department of LGH Lahore, provided valuable assistance for this research work which is thankfully acknowledged.

REFRENCES

1. Upadhyay M. P., Srinivasan M., Whitcher J. P. Microbial keratitis in the developing world: does prevention work?

International ophthalmology clinics, 2007; 1; 47 (3): 17-25.
2. Keay L., Edwards K., Naduvilath T., Taylor H. R., Snibson G. R., Forde K., Stapleton F. Microbial keratitis: predisposing factors and morbidity. Ophthalmology, 2006; 113 (1): 109-16.
3. Koo E. B., Colby K. Corneal diseases in children: infectious keratitis. InCorneal Diseases in Children, 2017; (pp. 13-38). Springer, Cham.
4. Sharma A., Nottage J. M., Mirchia K., Sharma R., Mohan K., Nirankari V. S. Persistent corneal edema after collagen cross-linking for keratoconus. American journal of ophthalmology, 2012; 154 (6): 922-6.
5. Insan N. G., Mane V., Chaudhary B. L., Danu M. S., Yadav A., Srivastava V. A review of fungal keratitis: etiology and laboratory diagnosis. Int. J. Curr. Microbiol. App. Sci., 2013; 2 (6): 307-14.
6. Bharathi M. J., Ramakrishnan R., Meenakshi R., Mittal S., Shivakumar C., Srinivasan M. Microbiological diagnosis of infective keratitis: comparative evaluation of direct microscopy and culture results. British Journal of Ophthalmology, 2006; 90 (10): 1271-6.
7. Dalmon C., Porco T. C., Lietman T. M., Prajna N. V., Prajna L., Das M. R., Kumar J. A., Mascarenhas J., Margolis T. P., Whitcher J. P., Jeng B. H. The clinical differentiation of bacterial and fungal keratitis: a photographic survey. Investigative ophthalmology & visual science, 2012; 53 (4): 1787-91.
8. Rathi V. M., Thakur M., Sharma S., Khanna R., Garg P. KOH mount as an aid in the management of infectious keratitis at secondary eye care centre. British Journal of Ophthalmology, 2017; 101 (11): 1447-50.
9. Zago V. V., Perez-Balbuena A. L. Laboratory in the Diagnosis of Bacterial and Fungal Keratitis. In Common Eye Infections, 2013; In Tech.
10. Zhang W., Yang H., Jiang L., Han L., Wang L. Use of potassium hydroxide, Giemsa and calcofluor white staining techniques in the microscopic evaluation of corneal scrapings for diagnosis of fungal keratitis. Journal of International Medical Research, 2010; 38 (6): 1961-7.
11. Kanski J. J., Bowling B. Clinical ophthalmology: a systematic approach. Elsevier Health Sciences; 2011.
12. Procop G. W. (ed.). Koneman's Color Atlas and Textbook of Diagnostic Microbiology, Philadelphia: Wolters Kluwer Health, 2017.
13. Cheesbrough M. District laboratory practice in tropical countries. Cambridge university press; 2006.
14. Tille P. Bailey & Scott's Diagnostic Microbiology-E-Book. Elsevier Health Sciences; 2013.
15. Orlans H. O., Hornby S. J., Bowler I. C. In vitro antibiotic susceptibility patterns of bacterial keratitis isolates in Oxford, UK: a 10-year review. Eye, 2011; 25 (4): 489.
16. DeCroos F. C., Garg P., Reddy A. K., Sharma A., Krishnaiah S., Mungale M., Mruthyunjaya P. Optimizing diagnosis and management of Nocardia keratitis, scleritis, and endophthalmitis: 11-year microbial and clinical overview. Ophthalmology, 2011; 118 (6): 1193-200.
17. Nayak N., Satpathy G., Prasad S., Titiyal J. S., Pandey R. M., Vajpayee R. B. Molecular characterization of drug-resistant and drug-sensitive Aspergillus isolates causing infectious keratitis. Indian journal of ophthalmology, 2011; 59 (5): 373.
18. Feilmeier M. R., Sivaraman K. R., Oliva M., Tabin G. C., Gurung R. Etiologic diagnosis of corneal ulceration at a tertiary eye center in Kathmandu, Nepal. Cornea, 2010; 29 (12): 1380-5.
19. Boggild A. K., Martin D. S., Lee T. Y., Yu B., Low D. E. Laboratory diagnosis of amoebic keratitis: comparison of four diagnostic methods for different types of clinical specimens. Journal of clinical microbiology, 2009; 47 (5): 1314-8.
20. Sharma S., Kunimoto D. Y., Gopinathan U., Athmanathan S., Garg P., Rao G. N. Evaluation of corneal scraping smear examination methods in the diagnosis of bacterial and fungal keratitis: a survey of eight years of laboratory experience. Cornea, 2002; 21 (7): 643-7.
21. Reddy A. K., Balne P. K., Gaje K., Garg P. PCR for the diagnosis and species identification of microsporidia in patients with keratitis. Clinical Microbiology and Infection, 2011; 17 (3): 476-8.