A Comparative Study for the Diagnosis of Microbial Keratitis Using Different Techniques

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Abstract: Purpose: The aim of the current study is to compare the sensitivity, specificity and predictive values of potassium hydroxide (KOH) wet mount with Gram stain versus culture procedures for the diagnosis of bacterial and fungal keratitis. **Methods:** One hundred and fifty patients clinically diagnosed as microbial keratitis who attended the Research Institute of Ophthalmology cornea clinic were investigated in our study. The samples collected were examined by direct microscopy using Gram stain as well as KOH wet mount and also plated on different culture media [sheep blood agar, chocolate agar, and Sabouraud's dextrose agar (SDA)]. The smear results were compared with culture findings of each case to investigate and analyze the sensitivity, specificity and predictive value of each technique using Medcalc program. **Results:** Gram stained smears showed positive bacterial growth in 121 patients out of the 150 studied cases with suspected ulcerative keratitis. A percentage of 72% (87 cases) out of these 121 cases was found to have positive bacterial growth in culture as well as in direct smear. Direct smear of Gram stained corneal scrapes showed fungal hyphae in only 4 cases, out of which 3 cases had also positive growth on SDA. On the other hand, using 10% KOH wet mount preparation for detection of fungal filaments illustrated that only 12 cases were positive, 9 of which turned out to be positive on SDA. The Gram stain smear technique reported significant higher sensitivity for detection of bacteria (84.47%) compared to that of fungal filaments (2.91%). However, the gram stained smear for the detection of fungal filaments illustrated significant higher specificity (97.87%) than that for the detection of bacteria (27.66%). The sensitivity of both the KOH wet mount preparation and Gram stain for the detection of fungal filaments was low (8.74% and 2.91% respectively) and correlated with higher specificity for both techniques (93.62% and 97.87% respectively). Positive predictive values were almost equal in both staining methods for the detection of fungal filaments. The negative predictive value was higher for Gram stain in the detection of bacteria (44.83%) than that for the detection of fungi (31.51%). The incidence percentage of bacteria in gram stained smears was 49.3 % (74 cases) among eyes with corneal ulcer larger than 2 mm and 2 % (3 cases) among eyes with corneal ulcer smaller than 2 mm**.** The incidence percentage of fungi in KOH smears was 12 % (18 cases) among eyes with corneal ulcer size larger than 2 mm in diameter and 0 % among eyes with corneal ulcer smaller than 2 mm size. **Conclusion:** Direct smear and culture techniques are of great diagnostic value for management of microbial keratitis. Direct smears with Gram stain are of higher diagnostic value in case of bacterial keratitis than in case of fungal keratitis; however, it is not as helpful on its own without confirmation with positive cultures for general diagnosis of cases of microbial keratitis.
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1. Introduction

Microbiology remains the critical tool in the diagnosis of microbial keratitis. Bacterial keratitis rarely occurs in the normal eye because of the human cornea's natural resistance to infection. However, a wide variety of conditions can lead to inflammation of the cornea. Among them are infections (viral, bacterial, or fungal); irritation from excessive use of contact lenses; dry eyes caused by tear film dysfunction. Exogenous factors such as systemic diseases and immuno suppression may alter the defense mechanisms of the corneal epithelium and permit organisms to invade the cornea (1,2). Culture and direct microscopic detection of causative organisms are the two important microbiological investigations that are widely used. Although culturing of microbial pathogens is considered to be the gold standard, direct microscopic evaluation of smears provides immediate information about the causative organisms. The conventional methods, potassium hydroxide (KOH) wet mount and Gram stain are widely used for the rapid detection of microbes (3,4); however, owing to misinterpretation, presence of arte facts, and lack of detection of *Candida* and other yeasts, the sensitivity of these methods is highly variable (3,5,6,7,8,9). Thus, there is a need to study the efficacy of available direct microscopic techniques in the detection of microbes from corneal scrapes, thereby creating an awareness to establish simple microbiological investigation in all ophthalmic clinics for timely treatment, and thereby preventing loss of vision. This study was conducted to evaluate microbial keratitis treated at the Research Institute of Ophthalmology, Cairo, Egypt. The aim of the current study is to compare the sensitivity, specificity and predictive values of potassium hydroxide

(KOH) wet mount with Gram stain versus culture procedures for the diagnosis of bacterial and fungal keratitis.

2. Materials and Methods:

Materials

Study Population

Our study included 150 (77 males and 73 females) patients with clinical evidence of microbial keratitis who attended the outpatient clinic corneal unit department of the Research Institute of Ophthalmology, Cairo, Egypt. Their age ranged from 2 to 83 years (mean 43 years). A total of 100 eyes of 50 patients were used as controls (30 males and 20 females), their age ranged from 25 to 50 years (mean 35 years). Control patients had normal ocular examination with no tear film dysfunction. A detailed history was taken and a thorough slit-lamp examination was done for all patients. Associated ocular conditions such as blepharitis, conjunctivitis, dacryocystitis, dry eyes and lid abnormalities were documented (10,11). The use of contact lenses and of topical corticosteroids and other systemic combinations were also recorded (10,11). In patients with infective keratitis the size, depth and margins of the infiltrate were noted. Satellite lesions and hypopyon were documented. Any epithelial defect was photographed and measured. Corneal scrapings were taken from the base and edge of the ulcers under aseptic techniques, with a sterile Bard-Parker blade (No 15), after installing local anesthetic solution (4% xylocaine) in the eye.

Methods

Scrap technique

The material obtained by scraping from the leading edge and the base of each ulcer was spread onto labeled slides in a thin, even manner for 10% potassium hydroxide (KOH) wet mount and Gram's staining (10,11,12,13). Also the scraping material obtained from each ulcer was inoculated directly onto sheep's blood agar, chocolate agar, and Sabouraud's dextrose agar (SDA) in a row of C-shaped streaks so that contamination could be spotted outside the C streaks and discarded. Deep inoculation in brain heart infusion broth was also done (10,11,12,13).

All inoculated media were incubated aerobically. The inoculated blood agar, chocolate agar, and brain heart infusion broth were incubated at 37°C, examined daily, and discarded at 5 days if no growth was seen (10). The inoculated Sabouraud's dextrose agar was incubated at 27°C, examined daily and discarded at 14 days if no growth was detected. Wilikins Chalgren anaerobic agar was incubated anaerobically in anaerobic jar at 37°C and examined after 5 days (14).

Following adequate growth of the fungal isolates on SDA, identification was done based on its macroscopic and microscopic features (14). To evaluate the diagnostic value of each assay, statistical analysis were done for calculating the sensetivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) using Medcalc program.

3. Results

From the 150 patients with clinical diagnosis of microbial keratitis it was found that 69.3% (104 cases) were culture positive. Of the culture positive cases (104); 51.3 % (77) had bacterial growth alone. 12% (18) had fungal growth alone. Six % (9) had mixed microbial growth. Fourty five point three % (68) had single strain of bacteria, 6% (9) had two strains of bacteria, 10.6% (16) had single strain of fungus, 1.3% (2) had two strains of fungi, 4.6% (7) had single strain of bacteria and single strain of fungus, 0.6% (1) had 2 strains of bacteria and single strain of fungus, While 30.6 % (46) were culture negative. The various isolates from cases of infective keratitis are shown in table (1). Gram positive cocci were predominantly isolated from the total number of bacterial cultures 66 species (77.21%). The microbial growth pattern of the corneal scrapings obtained from cases of infective keratitis and the percentage of various types of microbial species are shown in table (2). Table 3 shows the correlation between the direct smears and culture results of bacteria and fungus from corneal scrapes. From this table it was found that: Gram stained smears from corneal scrapes showed positive bacterial growth in 121 patients out of the 150 studied cases with clinically diagnosed microbial keratitis. A percentage of 72% (87 cases) out of these 121 cases was found to have positive growth in culture as well. Gram stained smears from corneal scrapes showed fungal hyphae in only 4 cases, out of which 3 cases had also positive growth. On the other hand, using 10% KOH wet mount preparation for detection of fungal filaments showed that only 12 cases were positive, 9 of which turned out to show positive growth. The Gram stain smear technique reported significant higher sensitivity for detection of bacteria (84.47%) compared to that of fungal filaments (2.91%). However, the gram stained smear for the detection of fungal filaments declared significant higher specificity (97.87%) than that for the detection of bacteria (27.66%). The sensitivity of both the KOH wet mount preparation and Gram stain for the detection of fungal filaments was low (8.74% and 2.91% respectively) and correlated with higher specificity for both techniques (93.62% and 97.87% respectively). Positive predictive values were almost equal in both staining methods for the detection of fungal filaments. The negative predictive value was higher for Gram stain in the detection of bacteria (44.83%) than that for the detection of fungi (31.51%).

Table 1: Various isolates from cases of microbial keratitis.

Bacteria:	No. of species= 94
Gram positive cocci	66 (70.21%)
Staphylococcus epidermidis	28 (29.78%)
Staphylococcus aureus	26 (27.65%)
Other staphylococci	2 (2.12 %)
pneumococci	5 (5.3 %)
Streptococcus viridans	3 (3.19%)
Gp A *streptococci*	1 (1.06%)
Micrococci	1 (1.06 %)
Gram positive bacilli	9
Corynebacterium	9 (9.57%)
Gram negative bacilli	12
Pseudomonas	10 (10.63 %)
Proteus	1 (1.06 %)
Acinetobacter	1 (1.06 %)
G –ve cocci	7
Neissereia spp. *Moraxella*	4 (4.25 %) 3 (3.1%)
Fungi:	No. of species=30
Aspergillus niger	7 (23.3 %)
Aspergillus flavus	7 (23.3 %)
Fusarium spp.	4 (13.3 %)
Rhizopus spp.	3 (10 %)
Rhodotorula	1 (3.3 %)
Mucor	1 (3.3%)
Candida	7 (23.3 %)

Table 4 shows the relation between size of corneal ulcer with the percentage of positive results of direct microscopic smear and culture for both bacteria and fungus: The incidence of gram smear positivity for bacteria was 49.3 % (74 cases) among eyes with corneal ulcer more than 2mm and 2 % (3 cases) among eyes with corneal ulcer below 2mm. Rate of positivity of Gram stain smear for fungus was 12 % (18 cases) among eyes with corneal ulcer size more than 2 mm in diameter and 0 % among eyes with corneal ulcer below 2 mm size. Also the incidence of gram smear positivity for fungus was 12 % (18 cases) among eyes with corneal ulcer more than 2mm and 0 % among eyes with corneal ulcer below 2mm.

Table 2: Microbial growth pattern of corneal scrapes obtained from 150 cases with infective keratitis

No	Growth pattern	No of cases (%)
1	Pure fungal growth	18 (12%)
	Single species of fungi	16 (88.8%)
	Two species of fungi	2 (11.1%)
2	Pure bacterial growth	77 (51.3%)
	Single species of bacteria	68 (88.3%)
	Two species of bacteria	9 (11.7%)
	More than two species o bacteria	0
3	Mixed fungal and bacterial growth	9
	Single species of fungi and single species of bacteria	7 (77.77%)
4	Eyes with microbial keratitis that showed positive cultures	104
5	Eyes with microbial keratitis that showed negative cultures	46

4. Discussion:

Methods for rapid detection of microbial agents and confirmation of clinical diagnosis are extremely important in the management of microbial keratitis. The common laboratory techniques for identifying microbial agents causing corneal infections are direct microscopic smear examinations of the corneal scrapes and culture (15,16). In addition, molecular diagnosis of pathogenic agents is a newer technology for accurate identification of the causative agents (17) but it is not easily applicable in all centers and its application is limited by its high cost. Cultures results may take up to 3 weeks depending on the causative organism. Direct microscopic examination of a corneal smear can provide results in a short span of time, enabling the clinician to start empirical treatment (18).

The material obtained from the smear was examined microscopically using Gram's stain and KOH. Gram's stain is a quick and helpful means of starting antibiotic therapy, 72% of the positive results with Gram stain were also culture positive thus one cannot rely completely on Gram stain alone. Owing to misinterpretation, presence of artifacts and lack of detection of *Candida* and other yeasts, the sensitivity of these methods is highly variable (3).

Table 3: Correlation between direct microscopic (10% KOH wet - mount preparation, Gram - stained) detection and culture - based diagnosis of fungus and bacteria from corneal scrapes obtained from 150 cases with infective keratitis.

Direct microscopic investigations	Results	No of cases	Culture		Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	Positive predictive value (%) (95% CI)	Negative predictive value (%) (95% CI)
			Positive	Negative				
Detection of fungal filaments in 10% KOH wet - mount preparation	Positive	12	9	3	8.74%	93.62%	75%	31.88 %
	Negative	138	94	44	4.07% to 15.94%	82.46% to 98.66%	42.81% to 94.51%	24.21% to 40.35%
	Total	150	103	47				
Detection of fungal filaments in Gram stained smear	Positive	4	3	1	2.91%	97.87%	75%	31.51%
	Negative	146	100	46	0.60% to 8.28%	88.71% to 99.95%	19.41% to 99.37%	24.08% to 39.71%
	Total	150	103	47				
Detection of bacteria in Gram stained smear	Positive	121	87	34	84.47%	27.66%	71.90%	44.83%
	Negative	29	16	13	76.00% to 90.85%	15.62% to 42.64%	63.01% to 79.69%	26.45% to 64.31%
	Total	150	103	47				

Table 4: Size of corneal ulcers evaluated in eyes and microbiological correlation.

Size of corneal ulcer	Positive for fungus						Positive for bacteria		
	KOH +ve	Gram +ve	Culture +ve	KOH and culture +ve	KOH and Gram +ve	Gram and culture +ve	Gram +ve	Culture +ve	Gram and culture +ve
<2 mm Early	0	0	0	0	0	0	3	3	3
2–>6 mm Advanced	0	18	18	0	0	18	74 (49.3%)	74 (49.3%)	74 (49.3%)
Total		18 (12%)	18 (12%)	0	0	18 (12%)	77 (51.3%)	77 (51.3%)	77 (51.3%)

Sharma *et al* (5) reported 81.2% sensitivity and 83.8% specificity of KOH wet-mount preparation in the detection of fungal filaments**. Hagan *et al* (18)** reported 80% sensitivity and 93% specificity of KOH preparation in fungal detection. However, in our study using KOH and gram stain for direct detection of fungal filaments, both gave a low sensetivity 8.74 % and 2.91 % respectively, while the specificity of the gram stain was higher than that of the KOH, 97.87 % and 93.62 % respectively.

The Gram stain smear technique reported significant higher sensitivity for detection of bacteria (84.47%) compared to that of fungal filaments (2.91%). However, the gram stained smear for the detection of fungal filaments illustrated significant higher specificity (97.87%) than that for the detection of bacteria (27.66%). **Gaudio *et al*. (17),** study the diagnosis of bacterial keratitis, the sensitivity of Gram stain (100%) was higher than that reported by Sharma *et al* (4) in early keratitis (36%) and also in advanced keratitis (40.9%). **Asbell and Stenson (19)** reported 67.0% sensitivity of Gram stain in the detection of bacteria in the US, and **Dunlop *et al* (20)** reported detection in Bangladesh. The results of this analysis indicate that Gram stain has a vital role in the diagnosis of bacterial keratitis. The size of the corneal ulcers, scraping technique, amount of the scraped material and microscopic observation of the scraped materials may be some of the factors that contributed to the results. In conclusion, after reviewing all available literature and preferred practice patterns, we recommend the practice

of routine microbiological analysis for all corneal ulcers.

However, unlike our study, **Whitcher *et al.* (16)** reported that there is a marked association between the smear positivity and size of the corneal ulcer. In our study, 147 out of 150 cases had corneal ulcer with size from 2mm-6mm at initial presentation indicating advanced stage where as 3 cases had corneal ulcer size below 2 mm indicating an early stage**.** Unlike other studies, in our study the size did not correlate significantly with the number of positive smears. Most of our large ulcers had associated melting which may limit the size of the sample. Out of the 147 advanced stage cases, 74 cases (49.3 %) had bacterial growth while 18 cases (12%) had fungal growth and 9 (6 %) cases had mixed bacterial and fungal growth.

We incubated our plates for microbial culture. The media were incubated under appropriate conditions, examined daily and require a specific period of time for positive growth depending on the organisms (24 hours to 3 weeks). A study by **O'Day *et al.* (8)** suggested that one-fourth of the fungal cultures did not become positive until 14-19 days after inoculation (4). Microbial cultures were considered relevant if growth of the same organism is observed on more than one solid-phase medium or if there is confluent growth at the site of inoculation on one solid medium or if growth of one medium is consistent with direct microscopy findings (i.e., appropriate staining and morphology with Gram's stain) and if the same organism is grown from repeated scraping. In patients who have received empirical therapy without doing routine microbiological analysis, a delay in starting culture-guided antibiotic treatment has been noted in a study by **Marangon *et al.* (21).** They reported that 56% of patients referred to their center were already on topical antibiotic therapy before culture specimen was obtained. If the cultures are negative, the ophthalmologist may consider stopping antibiotic treatment for 12-24 h and then re-culturing. In a study by **Kaye *et al.* (22)** there was no significant difference in the number of positive cultures from solid media (direct inoculation) used conventionally or liquid (indirect) culture media, both in patients and experimental pig corneas. The broth inoculation technique or the use of transport media (23) is a promising alternative to the recommended direct plate inoculation, especially in private eye clinics and community settings (24).

All patients were required to stop all antibiotics treatment 48 hours before samples were taken. In our study, positive cases were found to be much more in cultures inoculated in brain heart infusion broth 71cases (47.3%) , then blood agar 41 cases (27.3%), then chocolate agar 37 cases (24.6%), 27 cases (18%) illustrated positive growth on SDA and finally 20 cases (13.3%) were positive on Wilikns Chalgren anaerobic agar medium. In a previous study, a majority of their patients (92.5%) had corneal infection by a single agent, the most common being bacterial (51.9%). In our study, 69.3% (104 cases) of all 150 cases of suspected keratitis were culture positive. Of the culture positive cases (104): 51.3 % (77) had bacterial growth alone. 12% (18) had fungal growth alone. 6 % (9) had mixed microbial growth. 68 (45.3%) had single strain of bacteria. 6% (9) had two strains of bacteria. 10.6% (16) had single strain of fungus. 1.3%(2) had two strains of fungi. 4.6% (7) had single strain of bacteria and single strain of fungus. 0.6% (1) had 2 strains of bacteria and single strain of fungus. While 30.6 % (46 cases) showed no growth. In our study, bacterial keratites was predominantly caused by gram-positive bacteria. However, unlike other studies from Asia (25) and Africa (26) where infections by *Streptococcus pneumoniae* were most common; in our study, *Staphylococcus epidermidis*-related bacterial keratitis predominated. A review of literature showed that most of the studies from developed countries such as the USA (27) (except southern USA) and Australia (28) listed *S. epidermidis* or coagulase-negative staphylococci as the leading cause of bacterial keratitis. It is not clear whether the tendency to consider *S. epidermidis* or coagulase-negative *staphylococci* as a normal commensal of the conjunctiva may have led to underreporting in some of the studies. Nevertheless, the criteria to determine the significance of a positive culture from corneal scrapings appeared similar across most of these studies. Considering the fact that *S. epidermidis* forms the commonest commensal of the extraocular surfaces, it is highly probable that these organisms invade corneal tissues when compromised by antimicrobial and/ or corticosteroid therapy or trauma (27).

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