EXTENDED REPORT

Microbiological diagnosis of infective keratitis: comparative evaluation of direct microscopy and culture results

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Aims: To determine the sensitivity, specificity and predictive values of potassium hydroxide (KOH) wet mount, Gram stain, Giemsa stain and Kinyoun's acid-fast stain in the diagnosis of infective keratitis.
Methods: A retrospective analysis of all patients with clinically diagnosed infective keratitis presenting between September 1999 and September 2002 was carried out. Corneal scrapes were taken and subjected to direct microscopy and culture.
Results: 3298 eyes of 3295 consecutive patients with infective keratitis were evaluated, of which 1138 (34.51%) eyes had fungal growth alone, 1069 (32.41%) had bacterial growth alone, 33 (1%) had *Acanthamoeba* growth alone, 83 (2.5%) had mixed microbial growth and the remaining 975 (29.56%) had no growth. The sensitivity of KOH wet mount was higher (99.3%; 95% confidence interval (CI) 98.6 to 99.6) in the detection of fungi, 100% (95% CI 90.4 to 100) in the detection of *Nocardia* and 91.4% (95% CI 75.8 to 97) in the detection of *Acanthamoeba*) than that of Gram-stained smears (89.2% (95% CI 87.3 to 90.8) in fungi, 87% (95% CI 73.0 to 94.6) in *Nocardia* and 60% (95% CI 42.2 to 75.6) in the detection of *Acanthamoeba*) in the detection of fungi, *Nocardia* and *Acanthamoeba*. 1764 of 3295 (53.54%) patients presented more than 7 days after onset of illness and 84.69% of the eyes had corneal ulcers with size >2 mm in diameter. Positivities of KOH (44.46%; $p<0.001$) and Gram-stained smears (77.37%; $p<0.001$) were found to be higher among eyes with larger ulcers (>2 mm) than among eyes with smaller ulcers (<2 mm).
Conclusion: KOH smear is of greater diagnostic value in the management of infective keratitis, and it is recommended in all clinics without exception for establishing timely treatment.

Infective keratitis is an ocular emergency that requires prompt and appropriate management to ensure the best visual outcome for the patient. Without adequate treatment, corneal infection leads to blindness through corneal scarring and endophthalmitis.[1] To minimise ocular morbidity, timely antimicrobial treatment must be initiated on the basis of clinical and microbiological evaluation.[2 3] A clinical diagnosis of infective keratitis does not give an unequivocal indication of the causative organisms because a wide range of organisms can produce a similar clinical picture.[4–6] 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. Several techniques have been used for the direct microscopic identification of microbes from corneal scrapes: Gomori's methenamine silver,[7] periodic acid-Schiff,[8] calcofluor white[9] and fluorescein-conjugated lectins[10] yield accurate results, but are time consuming and require special infrastructures. In addition, the cost of each test makes them inapplicable in primary, secondary and even in most tertiary centres.

The conventional techniques, potassium hydroxide (KOH) wet mount, Gram stain and Giemsa stain, are widely used for the rapid detection of microbes[7 11]; however, owing to misinterpretation, presence of artefacts, and lack of detection of *Candida* and other yeasts, the sensitivity of these methods is highly variable.[7 9 12–15] 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 a simple microbiological investigation in all ophthalmic clinics for timely treatment, and thereby preventing loss of vision. This study was conducted to evaluate all microbial keratitis treated at a tertiary eye care referral centre in south India. The aims of this investigation were to determine the sensitivity and specificity of KOH smear, Gram stain, Giemsa stain and Kinyoun's acid-fast stain in the diagnosis of infective keratitis. We believe that these comprehensive data on direct microscopy will encourage ophthalmologists to carry out KOH smears even in small clinics.

MATERIALS AND METHODS

This retrospective study included all patients with clinically diagnosed infective keratitis presenting at Aravind Eye Hospital and Postgraduate Institute of Ophthalmology, Tirunelveli, south India over 3 years from September 1999 to September 2002. Corneal ulceration was defined as a loss of the corneal epithelium, with underlying stromal infiltration and suppuration associated with signs of inflammation with or without hypopyon.[16 17] Patients with suspected or confirmed viral keratitis and healing ulcers were excluded, as were those with Mooren's ulcers, interstitial keratitis, sterile neurotropic ulcers and any ulcer associated with autoimmune conditions. A standardised protocol was followed for each patient, with corneal ulceration for the evaluation of microbiological features and clinical findings.[16–18]

Clinical procedures

All patients were examined using a slit-lamp biomicroscope; the size of the epithelial defect after staining with 2% fluorescein was measured with the variable slit on the biomicroscope and recorded in millimetres. Using standard

techniques,[16–18] corneal scrape was observed under the magnification of a slit lamp or operating microscope after instillation of 0.5% proparacaine hydrochloride,[18] using flame-sterilised Kimura's spatula or a sterile Bard-Parker blade (no 15). The spatula or blade was scraped over the surface of the suppurative area in a series of short, moderately firm strokes to sample both the leading edges and the base of each infiltrated area in one direction from the peripheral margins towards the centre of the suppurative area. The material obtained was initially smeared onto clean sterile labelled glass slides for 10% KOH wet mount, Gram stain, Giemsa stain and Kinyoun's acid-fast stain for suspected actinomycete keratitis. The material obtained by the next scrape was inoculated directly onto the surface of solid media such as sheep's blood agar, chocolate agar, Sabouraud's dextrose agar, potato dextrose agar or non-nutrient agar in rows of C-shaped streaks, and also inoculated into the depth of liquid media such as brain heart infusion broth and thioglycollate medium. Rescrapes were taken after reflaming and cooling the spatula or using new sterile blades to obtain additional material from multiple areas of corneal suppuration.

Laboratory procedures

All inoculated media were incubated aerobically. The inoculated Sabouraud's dextrose agar was incubated at 27℃, examined daily, and discarded at 3 weeks if no growth was seen. The inoculated blood agar, chocolate agar, thioglycollate broth and brain heart infusion broth were incubated at 37℃, examined daily and discarded at 7 days if growth was not seen. The inoculated non-nutrient agar plates were incubated at 37℃ after overlaying with *Escherichia coli* broth cultures, were examined daily for the presence of *Acanthamoeba* species, and discarded at 3 weeks if there were no signs of growth.[16–18] Microbial cultures were considered relevant if growth of the same organism was observed on more than one solid-phase medium; if there was confluent growth at the site of inoculation on one solid medium; if growth of one medium was consistent with direct microscopy findings (ie, appropriate staining and morphology with Gram stain); or if the same organism was grown from repeated scraping.[16–18] Pearson's χ^2 test was used to carry out the statistical analysis wherever required and p value <0.05 was considered to be significant.

RESULTS

A total of 3295 patients with clinical diagnosis of infective keratitis were evaluated at our institute, of which a single eye was infected in 3292 patients and both eyes were infected in 3 patients; thus, a total of 3298 eyes with corneal ulceration were studied. Of 3298 eyes, 1138 (34.51%) eyes had fungal growth alone, 1069 (32.41%) had bacterial growth alone, 33 (1%) had *Acanthamoeba* growth alone, 83 (2.5%) had mixed microbial growth and the remaining 975 (29.56%) had no growth. Table 1 presents the microbial growth pattern of corneal scrapes obtained from 3298 eyes of patients with infective keratitis. A total of 1216 bacterial isolates from 1151 eyes with keratitis and 1226 fungal isolates from 1220 eyes with keratitis were recovered. Tables 2 and 3 document the bacterial and fungal pathogens respectively recovered from patients with infective keratitis.

Analysis using culture as the gold standard showed that the sensitivity of the KOH wet-mount preparation was higher (99.3% (95% confidence interval (CI) 98.6 to 99.6) in the detection of fungal filaments, 100% (95% CI 90.4 to 100) in the detection of *Nocardia* filaments and 91.4% (95% CI 75.8 to 97.8) in the detection of *Acanthamoeba* cysts) than that of Gram-stained smears (89.2% (95% CI 87.3 to 90.8) in the detection of fungal filaments, 87% (95% CI 73.0 to 94.6) in the detection of *Nocardia* spp and 60% (95% CI 42.2 to 75.6) in the detection of *Acanthamoeba* cysts) in the detection of fungi, *Nocardia* and *Acanthamoeba*, whereas the specificity of Gram-stained smears was 100% in the detection of fungi, *Nocardia* and *Acanthamoeba*. These results are summarised in table 4. The overall sensitivities of KOH smears (in the detection of fungus, *Nocardia* and *Acanthamoeba*) and Gram-stained smears (in the detection of fungus, bacteria (including *Nocardia*) and *Acanthamoeba*) were 99.1% (95% CI 98.3 to 99.5) and 93.8% (95% CI 92.8 to 94.7), respectively (table 5).

Table 1 Microbial growth pattern of corneal scrapes obtained from 3298 consecutive eyes with infective keratitis in south India

Sl no	Growth pattern	No of eyes (%)
1	Pure fungal growth	1138 (34.50)
	Single species of fungi	1132 (34.32)
	Two species of fungi	6 (0.18)
2	Pure bacterial growth	1069 (32.41)
	Single species of bacteria	1004 (30.44)
	Two species of bacteria	65 (1.97)
3	Pure protozoan (*Acanthamoeba*) growth	33 (1)
4	Mixed fungal and bacterial growth Single species of fungi and single species of bacteria	81 (2.46)
5	Mixed *Acanthamoeba* and single species of fungal growth	1 (0.03)
6	Mixed *Acanthamoeba* and single species of bacterial growth	1 (0.03)
	Eyes with microbial keratitis that showed positive cultures	2323 (70.44)
	Eyes with microbial keratitis that showed negative cultures	975 (29.56)

The false-negative rate of KOH smears was less (0.43% in the detection of fungi, 0% in the detection *Nocardia* and 0.09% in the detection of *Acanthamoeba*) than that of Gram-stained smears (5.97% in the detection of fungi, 0.18% in the detection of *Nocardia*, 0.43% in the detection of *Acanthamoeba*) and Giemsa-stained smears (0.58% in the detection of *Acanthamoeba*). The false-positive rate of KOH smears was 1.5% in the detection of fungi and 0% in the detection of *Nocardia* and *Acanthamoeba*, whereas no false-positive rate was noted for Gram-stained smears.

Most of the patients (53.54%) presented more than 7 days after onset of illness, and 100% of the patients with *Acanthamoeba* keratitis reported after 15 days. In all, 2793 of 3298 (84.69%) eyes had corneal ulcer with size >2 mm in diameter at initial presentation, of which 596 (18.07%) were >6 mm in diameter. Table 6 shows the size of the corneal ulcers measured and the duration of the patients' symptoms before presentation.

The rate of positivity of KOH smears for microorganisms was 44.6% among eyes with corneal ulcer size >2 mm in diameter and 13.06% among eyes with corneal ulcer <2 mm in size ($p<0.001$; odds ratio (OR) 5.33, 95% CI 4.0.4 to 7.04). Similarly, the incidence of Gram-stained smear positivity for microorganisms was 77.37% among eyes with corneal ulcer size >2 mm in diameter and 29.91% among eyes with corneal ulcer <2 mm in size ($p<0.001$; OR 8.02, 95% CI 6.46 to 9.95). Table 7 documents the size of the corneal ulcers evaluated and the microbiological correlation.

DISCUSSION

Methods for rapid detection of microbial agents and confirmation of clinical diagnosis are extremely important in the management of infective keratitis. The common laboratory techniques for identifying microbial agents causing corneal infections are culture and direct microscopic

Table 2 Bacterial pathogens isolated from corneal scrapes of 1151 eyes with infective keratitis treated at a tertiary eye care referral centre in south India

Sl no	Bacterial isolates	Pure isolates	Mixed with other bacteria	Mixed with fungal spp	Mixed with *Acanthamoeba* sp	Total no of bacterial isolates (%)
1	Gram-positive cocci	675	65	40		780 (64.14)
	Streptococcus pneumoniae	417	7	14		438 (36.03)
	Staphylococcus epidermidis	155	43	24		222 (18.25)
	Staphylococcus aureus	36	10	0		46 (3.78)
	Micrococcus spp	6	0	0		6 (0.49)
	α-Haemolytic streptococci	46	5	2		53 (4.36)
	β-Haemolytic streptococci	6		0		6 (0.49)
	Non-haemolytic streptococci	9		0		9 (0.74)
2	Gram-positive bacilli	33	22	2		57 (4.69)
	Bacillus spp	12	15	0		27 (2.22)
	Corynebacterium spp	21	7	2		30 (2.47)
3	Gram-negative cocci and coccobacilli	12				12 (0.99)
	Moraxella spp	9				9 (0.74)
	Neisseria spp	3				3 (0.25)
4	Aerobic actinomycetes	39	7			46 (3.78)
	Nocardia spp	39	7			46 (3.78)
5	Gram-negative bacilli	245	36	39	1	321 (26.40)
	Pseudomonas spp	173	29	36	1	239 (19.65)
	Enterobacter spp	26	5	3		34 (2.81)
	Klebsiella spp	10	2			12 (0.99)
	Proteus spp	6				6 (0.49)
	Alcaligens spp	6				6 (0.49)
	Haemophilus spp	6				6 (0.49)
	Acinetobacter spp	6				6 (0.49)
	E coli	4				4 (0.33)
	Serratia spp	3				3 (0.25)
	Citrobacter spp	5				5 (0.41)
	Total number of isolates (%)	1004 (82.57)	130 (10.69)	81 (6.66)	1 (0.08)	1216 (100)

smear examinations of the corneal scrapes.[16 19] In addition, molecular diagnosis of pathogenic agents is a newer technology for accurate identification of the causative agents[20] but is inapplicable to all patients with corneal ulcer, as it is more expensive. Although cultures require a longer time depending on the organisms (24 h to 3 weeks), examination of a smear can provide results in a short span of time, enabling the clinician to start empirical treatment.[21] At this study centre, we regularly carry out 10% KOH wet-mount preparation, Gram-stain procedure, and culture for all

Table 3 Fungal pathogens isolated from corneal scrapes obtained from 1220 eyes with infective keratitis treated at a tertiary eye care referral centre in south India

Sl no	Name of the fungal isolates	Pure isolates	Mixed with other fungal spp	Mixed with bacterial spp	Mixed with *Acanthamoeba* spp	Total number of fungal isolates (%)
1	Hyaline fungi	840	9	41	1	891 (72.68)
	Fusarium spp	483	3	24	1	511 (41.68)
	Aspergillus spp	292	4	9		305 (24.88)
	Mucor spp	6				6 (0.49)
	Rhizopus spp	4				4 (0.33)
	Penicillium spp	4				4 (0.33)
	Unidentified hyaline fungal species	51	2	8		61 (4.97)
2	Dematiaceous fungi	292	3	40		335 (27.32)
	Cladosporium spp	73	1	7		81 (6.61)
	Botryodiploidia spp	53		4		57 (4.65)
	Curvularia spp	43		9		52 (4.24)
	Biopolaris spp	26		3		29 (2.36)
	Exserohilum spp	21		3		24 (1.96)
	Alternaria spp	11		2		13 (1.06)
	Unidentified dematiacious fungal species	65	2	12		79 (6.44)
	Total number of isolates (%)	1132 (92.33)	12 (0.98)	81 (6.61)	1 (0.08)	1226 (100)

Table 4 Correlation between direct microscopic (10% KOH wet-mount preparation, Gram-stained, Giemsa-stained and Kinyoun's acid-fast stained smears) detection and culture-based diagnosis of fungus, bacteria, *Nocardia* and *Acanthamoeba* from corneal scrapes obtained from eyes (n = 3298) with infective keratitis

Sl no	Direct microscopic investigations	Results	No of eyes	Culture		Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	Positive predictive value (%) (95% CI)	Negative predictive value (%) (95% CI)
				Positive	Negative				
1	Detection of fungal filaments in 10% KOH wet-mount preparation	Positive	1230	1211*	19	99.3 (98.6 to 99.6)	99.1 (98.5 to 99.4)	98.5 (97.6 to 99.0)	99.6 (99.1 to 99.8)
		Negative	2068	9†	2059				
		Total	3298	1220‡	2078				
	Detection of fungal filaments in Gram-stained smear	Positive	1088	1088§	0	89.2 (87.3 to 90.8)	100 (99.8 to 100.0)	100 (99.6 to 100)	94 (92.9 to 95.0)
		Negative	2210	132†	2078				
		Total	3298	1220‡	2078				
2	Detection of bacteria in Gram-stained smear	Positive	1203	1151¶	52	100 (99.6 to 100)	97.6 (96.8 to 98.2)	95.7 (94.3 to 96.7)	100 (99.8 to 100)
		Negative	2095	0	2095				
		Total	3298	1151¶	2147				
3	Detection of *Nocardia* spp in 10% KOH wet-mount preparation	Positive	46	46**	0	100 (90.4 to 100)	100 (99.99 to 100)	100 (75.9 to 100)	99.4 (99.1 to 99.6)
		Negative	3252	0	3252				
		Total	3298	46**	3252				
	Detection of *Nocardia* spp in Gram-stained smear	Positive	40	40††	0	87 (73.0 to 94.6)	100 (99.9 to 100)	100 (89.1 to 100)	99.8 (99.6 to 99.9)
		Negative	3258	6‡‡	3252				
		Total	3298	46**	3252				
	Detection of *Nocardia* spp in Kinyoun's acid-fast stained smear	Positive	40	40††	0	87 (73.0 to 94.6)	100 (99.9 to 100)	100 (89.1 to 100)	99.8 (99.6 to 99.9)
		Negative	3258	6‡‡	3252				
		Total	3298	46**	3252				
4	Detection of *Acanthamoeba* spp in 10% wet-mount preparation	Positive	32	32§§	0	91.4 (75.8 to 97.8)	100 (99.9 to 100)	100 (86.7 to 100)	99.9 (99.7 to 100)
		Negative	3266	3¶¶	3263				
		Total	3298	35***	3263				
	Detection of *Acanthamoea* spp in Gram-stained smear	Positive	21	21†††	0	60 (42.2 to 75.6)	100 (99.9 to 100)	100 (80.8 to 100)	99.6 (99.3 to 99.8)
		Negative	3277	14¶¶	3263				
		Total	3298	35***	3263				
	Detection of *Acanthamoeba* spp in Giemsa-stained smear	Positive	16	16‡‡‡	0	45.71 (29.2 to 63.1)	100 (99.9 to 100)	100 (75.9 to 100)	99.4 (991 to 99.6)
		Negative	3282	19¶¶	3263				
		Total	3298	35***	3263				

*Of 1211 eyes, 1129 eyes had fungal growth alone, 81 eyes had both fungal and bacterial growth, and 1 eye had both fungal and acanthamoebic growth.
†Fungal growth alone.
‡Of 1220 eyes, 1138 had fungal growth alone, 81 had both fungal and bacterial growth, and 1 eye had both fungal and acanthamoebic growth.
§Of 1088 eyes, 1006 had fungal growth alone, 81 eyes had both fungal and bacterial growth, and 1 eye had both fungal and acanthamoebic growth.
¶Of 1151 eyes, 1069 eyes had bacterial growth alone, 81 eyes had both bacterial and fungal growth, and 1 eye had both bacterial and acanthamoebic growth.
**Of 46 eyes, 39 eyes had *Nocardia* growth alone and 7 eyes had both *Nocardia* and other bacterial growth.
††Of 40 eyes, 39 eyes had *Nocardia* growth alone and 1 had both *Nocardia* and other bacterial growth.
‡‡*Nocardia* growth alone.
§§Of 32 eyes, 30 eyes had acanthamoebic growth alone, 1 eye had both acanthamoebic and fungal growth, and 1 eye had both acanthamoebic and bacterial growth.
¶¶*Acanthamoeba* growth alone.
***Of 35 eyes, 33 eyes had acanthamoebic growth alone, 1 eye had both acanthamoebic and fungal growth, and 1 eye had both acanthamoebic and bacterial growth.
†††Of 21 eyes, 19 eyes had acanthamoebic growth alone, 1 eye had both acanthamoebic and fungal growth, and 1 eye had both acanthamoebic and bacterial growth.
‡‡‡Of 16 eyes, 14 eyes had acanthamoebic growth alone, 1 eye had both acanthamoebic and fungal growth, and 1 eye had both acanthamoebic and bacterial growth.

clinically diagnosed cases of infective keratitis. In addition, if required, Geimsa and Kinyoun's acid-fast staining procedures are also carried out for suspected cases. In this analysis, observations of smears and cultures of corneal scrapes obtained from all 3298 eyes highlight the value of the traditional method of KOH wet-mount preparation in the diagnosis of fungal, *Nocardia* and acanthamoebic keratitis. The overall sensitivity of KOH smears achieved in this study was higher than the sensitivity reported by other previous reports. Sharma *et al*[9] reported 81.2% sensitivity and 83.8% specificity of KOH wet-mount preparation in the detection of fungal filaments. Hagan *et al*[21] reported 80% sensitivity and 93% specificity of KOH preparation in fungal detection.

The size of the corneal ulcers, scraping technique, amount of the scraped material and microscopic observation of the scraped materials are some of the factors that contributed to this. Generally, corneal ulcers with large sizes will provide enough material for microbiological investigation, both for smears and cultures, and preparation of additional slides and inoculation of more than one culture media are also possible. In our centre, most patients (84.69%) with corneal ulcers presented with large ulcers (>2 mm in diameter) owing to late presentation. In this study, there is a marked association between the smear positivity and size of the corneal ulcer. Larger ulcers (>2 mm in diameter) provided a higher positivity rate in both KOH and Gram-stained smears than ulcers <2 mm in diameter, and thus the rate of positivity of smears increased according to the size of the corneal ulcers. Inappropriate previous medical treatment before presentation to our institute,[16] use of non-prescription drugs sold over the counter, use of traditional eye medicines,[16] non-availability of proper health eye care and poor socioeconomic status[19] are the important factors responsible for the late presentation of patients with corneal ulcers to tertiary eye centres.[16] This leads to increase in the size and severity of the ulcers, providing enough material with good microbial load for microscopy and culture. Hence, these factors may be responsible for increased sensitivity of the KOH smears in this study.

However, the value of 10% KOH wet-mount preparation in the diagnosis of fungal keratitis lies in its ability to clear the

Table 5 Overall efficacy of 10% KOH wet-mount preparation and Gram-stained smear in the detection of fungus, bacteria (including *Nocardia*) and *Acanthamoeba* from corneal scrapes obtained from eyes (n = 3298) with infective keratitis

Sl no	Direct microscopic investigations	Results	No of eyes	Culture		Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
				Positive	Negative				
1	Detection of fungus, *Nocardia* and *Acanthamoeba* in 10% KOH wet-mount preparation	Positive	1308*	1289†	19	99.1 (95% CI 98.3 to 99.5)	99.0 (95% CI 98.5 to 99.4)	98.5 (95% CI 97.7 to 99.1)	99.4 (95% CI 98.9 to 99.7)
		Negative	1990‡	12§	1978				
		Total	3298	1301¶	1997				
2	Detection of bacteria (including *Nocardia*), fungi and *Acanthamoeba* in Gram-stained smear	Positive	2352**	2300††	52	93.8 (95% CI 92.8 to 94.7)	93.9 (95% CI 92.0 to 95.3)	97.8 (95% CI 97.1 to 98.3)	83.9 (95% CI 81.4 to 86.2)
		Negative	946‡‡	152§§	794				
		Total	3298	2452***	846				

*Of 1308 eyes, 1230 were found to be positive for fungus, 46 were positive for Nocardia and 32 were positive for *Acanthamoeba* in 10% KOH wet-mount preparation.
†Of 1289 eyes, 1211 had fungal growth, 46 had Nocardia growth and 32 had *Acanthamoeba* growth.
‡Eyes negative for fungus, *Nocardia* and *Acanthamoeba* in KOH wet-mount preparation.
§Of 12 eyes, 9 had fungal growth and 3 had *Acanthamoeba* growth.
¶Of 1301 eyes, 1220 had fungal growth, 46 had *Nocardia* growth and 35 had *Acanthamoeba* growth.
**Of 2352 eyes, 1088 were found to be positive for fungus, 1203 were positive for bacteria, 40 were positive for *Nocardia* and 21 were positive for *Acanthamoeba* in Gram-stained smear.
††Of 2300 eyes, 1088 eyes had fungal growth, 1151 eyes had bacterial growth, 40 had *Nocardia* growth and 21 had *Acanthamoeba* growth.
‡‡Eyes negative for fungus, bacteria (including *Nocardia*) and *Acanthamoeba* in Gram-stained smear.
§§Of 152 eyes, 132 eyes had fungal growth, 6 had *Nocardia* growth and 14 had *Acanthamoeba* growth.
***Of 2452 eyes, 1220 had fungal growth, 1151 had bacterial growth, 46 had *Nocardia* growth, 35 had *Acanthamoeba* growth.

scrapes of cellular debris, thereby rendering hyphal fragments more refractile on microscopic examination. The staining quality of Gram stain is often variable, hyphal elements often appear as linearly stained precipitates, and it is usually not possible to determine whether they are coenocytic or septate. If the stained smear of scrape from an ulcer is thick, the hyphae will be interspersed throughout the necrotic tissue and their identification may be difficult or impossible.[22] Similarly, in KOH smears, *Nocardia* was easily recognised as very fine, intertwined, narrow, delicate, branching filaments, whereas in Gram-stained smears, *Nocardia* appeared as Gram-positive, beaded, coccoid and thin branching filaments, and were difficult to identify. In Kinyoun's acid-fast stained smears, *Nocardia* appeared as thin, pink, branching filaments on a blue background.[23] Although 10% KOH smear is a sensitive method for the detection of *Nocardia*, Kinyoun's acid-fast stain helps to differentiate *Nocardia* spp from *Actinomyces* spp by its acid-fastness.[23]

In the diagnosis of bacterial keratitis, the sensitivity of Gram stain (100%) obtained in this study was higher than that reported by Sharma *et al*[11] in early keratitis (36%) and also in advanced keratitis (40.9%). Asbell and Stenson[24] reported 67.0% sensitivity of Gram stain in the detection of bacteria in the US, and Dunlop *et al*[25] reported 62.0% detection in Bangladesh. The results of this analysis indicate that Gram stain has a vital role in the diagnosis of bacterial keratitis. Similar to fungi and *Nocardia* detection, the sensitivity of 10% KOH smears (91.45%) was higher than that of Gram-stained smears (60%) and Giemsa-stained smears (45.71%) in the detection of *Acanthamoeba*. Sharma *et al*[26] reported 87.1% sensitivity of KOH with calcofluor white preparation in the detection of *Acanthamoeba* cysts in Hyderabad. In the 10% KOH wet-mount preparation, *Acanthamoeba* was recognised in the form of double-walled cysts, having an outer wrinkled wall (ectocysts) and an inner polygonal, stellate wall (endocyst).[27] The clarity of the cysts in the preparation was remarkable. However, in older KOH preparations, slight disintegration was noted in the cyst morphology. The staining quality of Gram-stained smears is often variable. In Gram-stained smears, the structure of the cyst was not as remarkable, and resembled tissue macrophages, mononuclear cells or degenerative epithelium.

Although the rate of positivity of Gram-stained smears seemed to be higher than that of KOH smears in the detection of microorganisms, the overall analysis shows that the sensitivity of KOH smears was markedly higher than that of Gram-stained smears in the detection of fungi, *Nocardia* and *Acanthamoeba*. If we carry out KOH smears alone, we can easily classify corneal ulcers based on the result of KOH smears as fungal, *Nocardia* or *Acanthamoeba*, where the smear would be positive for these organisms, or other than these three groups where it would be negative, and this is helpful to institute early, specific treatment. Thus, a KOH smear is of

Table 6 Size of corneal ulcers (n = 3298) and duration of symptoms before presentation, and microbial growth pattern of patients (n = 3295) with infective keratitis

Sl no	Demographic characters		Total no of cases (%)	Bacterial growth (%)	Fungal growth (%)	*Acanthamoeba* growth (%)	Bacterial and fungal (%)	*Acanthamoeba* and fungal (%)	*Acanthamoeba* and bacterial (%)	No growth (%)
1	Size of the corneal ulcer	<2 mm	505 (15.31)	109 (10.2)	59 (5.19)	0	0	0	0	337 (34.56)
		2–6 mm	2197 (66.62)	797 (74.55)	785 (68.98)	6 (18.18)	17 (20.99)	0	0	592 (60.72)
		>6 mm	596 (18.07)	163 (15.25)	294 (25.83)	27 (81.82)	64 (79.01)	1 (100)	1 (100)	46 (4.72)
	Total no of eyes studied		3298 (100)	1069 (100)	1138 (100)	33 (100)	81 (100)	1 (100)	1 (100)	975 (100)
2	Duration of presentation after onset of illness	1–3 days	497 (15.08)	247 (23.17)	109 (9.58)	0	0	0	0	141 (14.46)
		4–7 days	1034 (31.38)	331 (31.05)	415 (36.47)	0	5 (6.17)	0	0	283 (29.02)
		8–14 days	817 (24.8)	264 (24.76)	370 (32.51)	0	19 (23.46)	0	0	164 (16.82)
		15–29 days	541 (16.42)	105 (9.85)	111 (9.75)	2 (6.06)	39 (48.15)	0	0	284 (29.13)
		1–2 months	225 (6.83)	91 (8.54)	69 (6.06)	16 (48.48)	12 (14.81)	1 (100)	1 (100)	35 (3.59)
		>2 months	181 (5.49)	28 (2.63)	64 (5.62)	15 (45.45)	6 (7.41)	0	0	68 (6.97)
	Total no of patients studied		3295 (100)	1066 (100)	1138 (100)	33 (100)	81 (100)	1 (100)	1 (100)	975 (100)

Table 7 Size of corneal ulcers evaluated in 3298 eyes and microbiological correlation

Sl no	Size of corneal ulcer	Positive for fungus						Positive for bacteria including *Nocardia*			Positive for *Acanthamoeba*						Positive for *Nocardia*					
		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	KOH +ve	Gram +ve	Culture +ve	KOH and culture +ve	KOH and Gram +ve	Gram and culture +ve	KOH +ve	Gram +ve	Culture +ve	KOH and culture +ve	KOH and Gram +ve	Gram and culture +ve
1	<2 mm	61	15	59	50	15	15	136	109	109	0	0	0	0	0	0	5	0	5	0	0	0
2	2–6 mm	806	718	802	802	719	719	826	814	814	5	0	6	5	0	0	23	22	23	22	22	22
3	>6 mm	363	355	359	359	355	355	241	228	228	27	21	29	27	21	21	18	18	18	18	18	18
	Total	1230	1088	1220	1211	1088	1088	1203	1151	1151	32	21	35	32	21	21	46	40	46	40	40	40

greater diagnostic value in the management of infective keratitis, and so the simple, sensitive technique of KOH wet mounting is recommended in all clinics without exception for establishing timely treatment. This study reports on examination of smears of corneal scrape results for fungi, bacteria, *Nocardia* and *Acanthamoeba*. Gram stain was found to be very dependable for making decisions in the treatment of bacterial keratitis, thereby ensuring a place for empirical treatment in suspected cases of bacterial keratitis. KOH wet-mount preparation was highly reliable in confirming the diagnosis of fungal keratitis, *Nocardia* keratitis and *Acanthamoeba* keratitis.

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REFERENCES

1 **Whitcher JP**, Srinivasan M, Upadhyay MP. Corneal blindness: a global perspective. *Bull WHO* 2001;**79**:214–21.
2 **Jones DB**. Decision making in the management of microbial keratitis. *Ophthalmology* 1981;**88**:814–20.
3 **Allen BDS**, Dart JKG. Strategies for the management of microbial keratitis. *Br J Ophthalmol* 1995;**79**:777–86.
4 **Sridhar MS**, Gopinathan U, Garg P, *et al*. Aspergillus fumigatus keratitis with wreath pattern infiltrates. *Cornea* 2001;**20**:534–5.
5 **Ostler HB**. *Diseases of the external eye and adnexa*. Baltimore: Williams &Wilkins, 1993:173–91.
6 **Florakis GJ**, Moasami G, Schubert H, *et al*. Scanning slit confocal microscopy of fungal keratitis. *Arch Ophthalmol* 1997;**115**:1461–3.
7 **Liesegang TJ**, Forster RK. Spectrum of microbial keratitis in South Florida. *Am J Ophthalmol* 1980;**90**:38–47.
8 **Xie L**, Dong X, Shi W. Treatment of fungal keratitis by penetrating keratoplasty. *Br J Ophthalmol* 2001;**85**:1070–4.
9 **Sharma S**, Silverberg M, Mehta P, *et al*. Early diagnosis of mycotic keratitis: predictive value of potassium hydroxide preparation. *Indian J Ophthalmol* 1998;**46**:31–5.
10 **Robin JB**, Nielson S, Tronsdale MD. Fluorescein-conjugated lectin identification of a case of human keratomycosis. *Am J Ophthalmol* 1986;**102**:797–8.
11 **Sharma S**, Kunimoto DY, Gopinathan U, *et al*. 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**:643–7.
12 **Rosa RH**, Miller D Jr, Alfonsa EC. The changing spectrum of fungal keratitis in South Florida. *Ophthalmology* 1994;**101**:1005–13.
13 **Rao NA**. A laboratory approach to rapid diagnosis of ocular infections and prospects for the future. *Am J Ophthalmol* 1989;**107**:283–91.
14 **O'Day DM**, Burd EM. Fungal keratitis and conjunctivitis: mycology. In: Smolin G, Thoft RA, eds. *The cornea: scientific foundations and clinical practice*. 3rd edn. Boston: Little, Brown & Co, 1996:229–39.
15 **Vajpayee RB**, Angra SK, Sandramouli S, *et al*. Laboratory diagnosis of keratomycosis: comparative evaluation of direct microscopy and culture results. *Ann Ophthalmol* 1993;**25**:68–71.
16 **Srinivasan M**, Gonzales CA, George C, *et al*. Epidemiology and aetiological diagnosis of corneal ulceration in Madurai, South India. *Br J Ophthalmol* 1997;**81**:965–71.
17 **Wilhelmus KR**, Liesegang TJ, Osato MS, *et al*. Laboratory diagnosis of ocular infections. In: Specter SC, eds. *CUMITECH*. Washington, DC: American Society for Microbiology, 1994.
18 **Brinser JH**, Burd EM. Principles of diagnostic ocular microbiology. In: Tabbara KF, Hyndiuk RA, eds. *Infections of the eye*. 2nd edn. Boston: Little, Brown & Co, 1996:69–84.
19 **Whitcher JP**, Srinivasan M, Upadhyay MP. Microbial keratitis. In: Johnson GJ, Minassian DC, Weale RA, West SK, eds. *The epidemiology of eye diseases*. 2nd edn. London: Arnold, 2003:190–5.
20 **Gaudio PA**, Gopinathan U, Sangwan V, *et al*. Polymerase chain reaction based diagnosis of fungi in infected corneas. *Br J Ophthamol* 2002;**86**:755–60.
21 **Hagan M**, Wright E, Newman M, *et al*. Causes of suppurative keratitis in Ghana. *Br J Ophthalmol* 1995;**79**:1024–8.
22 **Wilson LA**, Sexton RR. Laboratory diagnosis in fungal keratitis. *Am J Ophthalmol* 1968;**66**:646–53.
23 **Gordon MA**. Aerobic pathogenic Actinomycetaecae. In: Lennette EH, Balows A, Hausler WJ Jr, Shadomy HJ, eds. *Manual of clinical microbiology*. 4th edn. Washington, DC: American Society for Microbiology, 1985:249–62.
24 **Asbell P**, Stenson S. Ulcerative keratitis. Survey of 30 years laboratory experience. *Arch Ophthalmol* 1982;**100**:77–80.
25 **Dunlop AA**. Wright ED, Howlader SA, *et al*. Suppurative corneal ulceration in Bangladesh. A study of 142 cases examining the microbial diagnosis, clinical and epidemiological features of bacterial and fungal keratitis. *Aust NZ J Ophthalmol* 1994;**22**:105–10.
26 **Sharma S**, Grag P, Roa GN. Patients characteristics, diagnosis and treatment of non-contact lens related *Acanthamoeba* keratitis. *Br J Ophthalmol* 2000;**84**:1103–8.
27 **Illingworth CD**, Cook SD. *Acanthamoeba* keratitis. *Surv Ophthalmol* 1998;**42**:493–508.