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Evaluation of impression cytology as rapid non-invasive diagnostic tool in infected corneal ulcer

Saraa Mohamed Abdel Rahman Elsheikh
Ministry of Health (ophthalmology specialist in Tanta Ophthalmic Hospital)

Kareman Ahmed Ebrahim Eshra
Assistant prof of Microbiology and Immunology, Faculty of Medicine, Tanta University, Tanta, Egypt
ORCID 0000-0002-5742-3843
Corresponding author email: drkaremaneshra2004@hotmail.com

Khaled Ahmed Nagy
Ophthalmology, Departments, Faculty of Medicine, Tanta University, Egypt

Moataz Mohamed Sabry
Ophthalmology, Departments, Faculty of Medicine, Tanta University, Egypt

Adel Abdou Selima
Ophthalmology, Departments, Faculty of Medicine, Tanta University, Egypt

***Abstract*---**Background: Microbial keratitis (MK) is caused by bacteria, fungi, and protozoa. It is very important to differentiate between these three causative microorganisms very early as they differ in their treatment, and some of these microorganisms, like *Pseudomonas aeurogenosa*, may result in eye perforation within 72 hours .The purpose of this study was to evaluate impression cytology smear to be used as a guide for early accurate treatment of cases. This study was conducted on 80 patients suspected of having MK. A corneal smear was taken with impression cytology paper (a non-invasive technique) and transported to the microbiology laboratory for examination. Results: The IC smear examination results were as follows: 30 (37.5%) were *Aspergillus fungus*, 14 (17.5%) were *Acanthamoeba*, 15 (18.75%) were *Gram-negative bacilli*, 4 (5%) were *Staphylococci*, 1 (1.25%) was *Streptococci*, 14 (17.5%) were both *Gram-negative bacilli* and *Aspergillus fungus*, and 2 (2.5%) were both *Staphylococci* and *Aspergillus fungus*. There was no significant difference between the diagnosis of MK by the culture method and the IC smear method.

Conclusion: The impression cytology smear can be used as guide for early treatment of MK.

***Keywords*---**corneal ulcer, microbial keratitis, impression cytology, smear, culture, non-invasive diagnosis.

Introduction

Infected corneal ulcers, called MKs, are one of the most important infectious eye diseases and may result in monocular blindness in some cases (Ting et al., 2021). Contact lenses are a common risk factor for ulcerative MK (Moshirfar et al., 2019). As a result, early and rapid diagnosis of MK has a significant impact on its morbidity and mortality rates (Hoffman et al., 2022).MK can be caused by bacteria, fungi, protozoa, and viruses (Zemba et al., 2022). The most common causative bacteria for MK are gramme-positive cocci such as Staphylococcus aureus, Streptococcus pneumoniae, and Staphylococcus epidermidis. Gram-negative bacilli such as Pseudomonas aeruginosa, Acinetobacter species, and Gram-negative coccobacilli such as Hemophilus influenza and Haemophilus aegypti (Gurnani et al., 2022). Aspergillus, Fusarium, and Candida species are the most common causative fungi for MK (Castano et al., 2021). Acanthamoeba is the most common causative protozoa for MK (Rezaei et al., 2013). The clinical examination and risk factors for MK may help in the prediction of the causative organism but may not be accurate enough, and the clinicians must know the causative organism for an accurate prescription of treatment (Hoffman et al., 2022).The gold standard for detection of causative microorganisms in MK is still culture, but it is time-consuming (Zemba et al., 2022). As a result, we require a method for diagnosing MK that is as accurate as culture but more rapid (Hoffman et al., 2021). Also, the culture methods are weak diagnostic tools for fungal and protozoal MK (Patel et al., 2016). Confocal microscopy and PCR can be used for the diagnosis of MK, but these methods are very expensive. Corneal scraping and impression cytology can be used for sampling in the diagnosis of MK, but impression cytology sampling is a more easy and non-invasive technique. (Alkatan et al., 2019, Goh et al., 2018). Many studies consider smear and culture as the basic standard diagnostic methods for MK (Tananuvat et al., 2012, Shabrawy et al., 2013). Another important benefit of the culture is testing for antibiotic sensitivity, but we cannot wait for its results to start the medical treatment; corneal perforation in fungal keratitis can occur in a few days, and culture results take days to appear; also, corneal melting in acanthamoebic keratitis and Pseudomonas keratitis occurs very rapidly [Rai et al., 2016 , Bharathi et al., 2006, Kaye et al., 2016]. As a result, some clinicians rely on provisional diagnosis in MK diagnosis based on clinical examination and risk factor expectations and initiate empirical treatment with multiple drugs without waiting for microbiological laboratory investigation, which may result in corneal toxicity, delayed epithelial healing, and aggravate the progression of destructive processes in the MK such as fungal keratitis perforation and Pseudomonas keratitis melting of the cornea. Because of nonspecific empirical treatment to avoid the delay of culture results before starting specific treatment, MK has become a disease with high morbidity and mortality rates (Moe et al., 2022, Somerville et al., 2021). So, the aim of this study was to implement early

differential diagnosis of MK in order to stop using an empirical treatment strategy in the management of MK. Moreover, to evaluate the IC smear as a non-invasive, accurate, economical, and rapid method able to differentiate between the three types of causative microorganisms of MK (bacterial, fungal, and protozoal causes of MK). Finally, to encourage ophthalmologists to start specific treatment and stop the empirical treatment strategy, as the three common causes of MK mentioned above differ greatly in their treatments.

Patients and Methods

Patients

This study was carried out in the Department of Medical Microbiology and Immunology, Faculty of Medicine, Tanta University, on 80 patients with suspected MK from the Ophthalmology Department, Faculty of Medicine, and Tanta University's outpatient clinic between October 2020 and September 2021. Written consents were obtained from all the patients in this research or their close relatives if the patients were uncooperative. Ethical approval for this study was provided by the Ethics and Research Committee, Faculty of Medicine, Tanta University. Inclusion criteria were patients with suspected MK caused by bacteria, fungi, or acanthamoeba from history and clinical examination. Exclusion criteria were patients with viral keratitis, patients who received previous empirical treatment, and patients with a non-infected corneal ulcer known as Mooren's ulcer.

Methods

All patients included in the study were subjected to the following:

2.2.1. Taking a history: Cases were found in the outpatient clinics of the Ophthalmology Department, Faculty of Medicine, and Tanta University Hospital complaining of rapid and painful progressive loss of vision with a history of predisposing factors such as contact lens abusers, eye trauma, particularly with plant objects, immunocompromised patients, and chronic use of steroid or anesthetic eye drops.
2.2.2. Clinical examination of the cornea using a slit lamp
2.2.3. Microbiological examination (Cheesbrough, 2006):
2.2.3.1. Samples: Impression cytology swabs were taken and transported to the microbiology laboratory in brain-heart infusion broth bottles (a non-invasive process).
2.2.3.2. Sample processing: The glass bottle was vortexed for 5–10 s, and then one sterilized disposable swab was used for every sterilized glass slide to spread the smear on the slide. Microscopic examination: The first slide was mixed with distilled water to make the wet film and examined under light microscopy with low- and high-power lenses to detect the amoeba. The second slide was mixed with lactophenol cotton blue stain and examined under light microscopy with low- and high-power lenses to detect the fungal hyphae; a third slide was stained with gram stain and examined under an oil immersion lens to detect different causative bacteria. Three swabs were cultured on Blood, MacConkey's, and Sabouraud's dextrose agar culture media and then incubated at 37^{o} C in the

incubator for 24 hours for Blood and MacConkey's and 48 hours for Sabouraud's dextrose agar; the media were not discarded if they gave us no growth except after 3 weeks of incubation; colonies were identified by colony morphology and a lactophenol cotton.

Statistical Analysis

Statistical analysis was performed using the GraphPad Prism software. Qualitative data are described as numbers and percentages. Pearson Chi-square tests were used to compare groups as appropriate. (P 0.05 was considered a significant result).

Results

This study included 80 patients from the outpatient clinic of the ophthalmology department at Tanta University, faculty of medicine. 48 cases (60%) were male, and 32 cases (40%) were female. The patients' ages ranged from 17 to 81.18 patients gave a definite history of eye trauma with vegetative matter; 12 patients gave a history of eye trauma; 24 patients gave a history of wearing contact lenses; 12 patients were immunocompromised; 10 patients were abusers of eye drops (topical anaesthesia or topical steroids); and 4 patients gave a history of extra-ocular surgeries (pterygium excision and squint surgery).

Table 1: Results of the diagnosis of microbial keratitis with clinical examination, impression cytology (IC) smear, and culture method (gold standard test).

		N	%
Clinical examination	Pure fungal keratitis	20	25.0
	Pure bacterial keratitis	26	32.5
	Mixed keratitis (both fungal and bacterial)	26	32.5
	Acanthameba	8	10.0
	Total	80	100
Smear	Lactophenol Cotton blue stain	30	37.5
	Gram stain	20	25.0
	Wet film	14	17.5
	both Gram and lactophenol stains	16	20.0
	Total	80	100
Culture	Sabaroud dextrose agar culture	26	32.5
	Blood - MacConkey's agar culture	22	27.5
	Blood, MacConkey's and sabaroud's dextrose agar	18	22.5
	No culture	14	17.5
	Total	80	100

Table 1 shows the provisional diagnosis of microbial keratitis by clinical examination with slit-lamp corneal examination and the incidence of each microbial type in the study cases: 20 cases (25%) had pure fungal keratitis, 26

cases (32.5%) had pure bacterial keratitis, 26 cases (32.5%) had mixed (bacterial and fungal) keratitis, and 8 cases (10%) had acanthamoebic keratitis. The results of an impression cytology (IC) smear test with different stains were as follows: IC smears with lactophenol cotton blue stain were positive in 30 cases (37.5%), IC smears with Gram stain were positive in 20 cases (25%), IC wet films were positive in 14 cases (17.5%), and both Gram and lactophenol cotton blue stains were positive in 16 cases (20%). Sabaroud's dextrose agar cultures were positive in 26 cases (32.5%), Blood and MacConkey's agar cultures were positive in 22 cases (27.5%), 18 cases were positive in all three cultures as mixed keratitis (22.5%), and 14 cases were negative in all three cultures because they were diagnosed with acanthamebic keratitis with wet film only.

Table 2: Comparison between the clinical examination results and both the culture and IC smear results

clinical examination	Culture		Smear	
	N	%	N	%
Bacterial keratitis 26(32.5%)	22	27.5	20	25
Fungal keratitis 20(25%)	26	32.5	30	37.5
Mixed keratitis 26(32.5%)	18	22.5	16	20
Acanthamebic keratitis 8(10%)	-	-	14	17.5
Total	66	82.5	80	100
X^2	14.031		6.804	
P value	0.001*		0.042*	

* Significant

Table 2 showed that there was a significant difference between the clinical examination results and both the culture and IC smear results in the diagnosis of microbial keratitis as P values were significant.

Table 3: Comparison between IC smear results and culture results

	Culture		Smear		P value
	N	%	N	%	
Bacterial keratitis	22	27.5	20	30.3	0.710
Fungal keratitis	26	32.5	30	37.5	0.109
Mixed keratitis	18	22.5	16	24.2	0.804
Acanthamebic keratitis	-	-	14	17.5	
Total	66	82.5	80	100	

Table 3 showed that there was a non-significant difference between the culture results and the smear results in the diagnosis of microbial keratitis; the p values were non-significant.

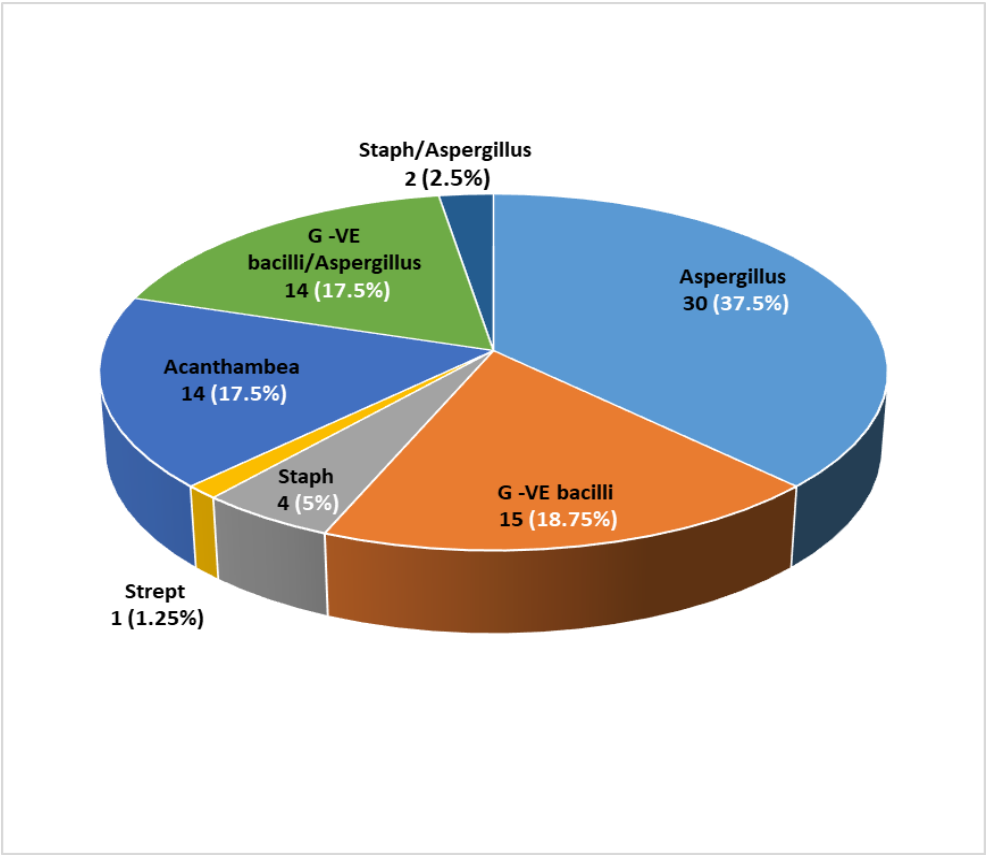

Figure 1. Results of the IC smear examination

Figure 1 shows the results of the IC smear examination as follows: 30 (37.5%) were *Aspergillus fungus*, 14 (17.5%) were *Acanthamoeba*, 15 (18.75%) were *Gram-negative bacilli*, 4 (5% were *Staphylococci*), 1 (1.25% was *Streptococci*), 14 (17.5%) were both *Gram-negative bacilli* and *Aspergillus fungus*, and 2 (2.5% were both *Staphylococci* and *Aspergillus fungus*).

Discussion

Table 1 displayed the results of MK diagnosis using the three methods used in our study, and Figure 1 displayed the result of IC smear examination, which revealed that the most common bacterial cause of MK was *gram negative bacilli*, followed by *Staphylococci* and *Streptococci*, and the IC smear was able to differentiate and detect these three different bacterial causes of MK, and this differentiation is very important to the clinician and greatly influences their decision in treating patients. Sharma et al. (2007) stated that most cases of MK were misdiagnosed based solely on clinical examination and treated empirically. Also, Lin et al. (2019) detected that the majority of community-acquired cases of bacterial keratitis are treated with empirical therapy and managed without smears or cultures. This is explained by the fact that most cases of MK in the USA are pure bacterial and respond well to broad-spectrum antibiotics, but in our tropical countries, fungal keratitis and mixed keratitis have the highest incidence,

which is worsened if we start with empirical treatment and change its characteristic signs with antibiotics, which also increase the virulence of microorganisms and delay the recovery of cases. In the current study, the impression cytology membrane had been used in corneal smear sample collection as it has a lot of advantages over the scraping technique, including: the sample can be taken without slit-lamp or surgical microscopy, which is in agreement with Jain et al., (2007) and Thia et al., (2019) but Somerville et al., (2021) Moshtaghion et al., (2020) ,Jain et al., (2007) ,and we agree with this point of view that impression cytology is neither an invasive nor traumatizing technique in an uncooperative patient with a painful, weak, friable, and thin cornea (Moshtaghion et al., 2021, Salmon et al., 2015). Also, multiple samples can be taken without worry of corneal perforation (Thia et al., 2019). Although most research on the scraping technique comments on the importance of lesion size being > 2 mm, IC can take accurate samples from smaller lesions, and this agrees with Jain et al. (2007). It also covers a larger area of a corneal ulcer. Lastly, another advantage of the membrane over the scraping technique is that the membrane has the printing property, which does not crash the sample tissue as does scraping, giving more accurate results not only in microscopic examination but also in PCR Jain et al. (2007).

There were many types of stains that could be used for the diagnosis of MK at the level of the smear stain (Salmon et al., 2015). Rathi et al., 2021 mentioned that direct examination of corneal scraping smears using different stains (KOH 10%, calcofluor white, and Gram stains) is part of the standard protocol for the laboratory diagnosis of non-viral keratitis. Also, Sharma et al. (2007) detected that the initial smear examination helped in instituting specific therapy and improving the outcome in cases of MK. According to Bharathi et al. (2006) , a smear is a reliable test for initiating specific medical treatment until culture results confirm the diagnosis. Also, Shabrawy et al. (2013) strongly recommend the use of direct microscopy of a corneal smear as a rapid, economic, and sensitive method for screening for fungal keratitis. But Shemis and Rahman (2012) , Fanselow et al. (2021) , and others concluded that direct smear and culture techniques are of great diagnostic value for the management of MK. Direct smears with Gram stain are of higher diagnostic value in cases of bacterial keratitis than fungal keratitis; however, they are not as helpful on their own without confirmation with positive cultures for the general diagnosis of cases of MK. Sharma et al. (2007) recommended that all suspected cases of MK be scraped for a smear and culture before initiating medications. Also, Hoffman et al. (2022) mentioned that smear microscopy alone was not considered to be conclusive evidence if only a few organisms were seen. From the results of Table 1 of this current study, the fungal keratitis was considered to have the highest incidence of microbial keratitis due to the humid and hot climate in Egypt and the agricultural environment, which increase the incidence of trauma in vegetative matters, and these results were in agreement with the Egyptian research of Shabrawy et al., (2013) and Sharma et al., (2007) , who did their research in India, which has similar economic conditions, a tropical area, and a climate similar to Egypt. Jain et al. (2007) reported that the sensitivity of the impression smear technique in comparison to conventional mechanical corneal scraping was 97.14%, the specificity of the technique was 92.86%, the positive predictive value of impression cytology was 94.44%, the negative predictive value was 92.86%, and the accuracy was 68%.

They concluded that the impression smear KOH examination is comparable to the conventional mechanical corneal scraping KOH examination in making a tentative diagnosis of fungal keratitis and can be accurately relied upon for initiating anti-fungal therapy. So, like corneal scraping, an IC smear is an accurate method, but it is non-invasive and does not require a slit lamp examination. Culture is still the gold standard in the diagnosis of microbial keratitis. This is in agreement with Hoffman et al., 2022, and Alkatan et al., 2019. Shabrawy et al. (2013) compared PCR with direct smear microscopy and culture in fungal keratitis in Egypt; she demonstrated that fungal culture positivity was 55% of the study cases and that it takes up to 4 weeks to be positive; she concluded that PCR can detect fungi in a high proportion of culture-negative cases, but it is difficult to use as a routine diagnostic test due to economic reasons. In our study, we diagnosed Acanthamoebic keratitis by clinical examination and wet film only, and when we compared the clinical examination with both the culture method and the smear method in the diagnosis of MK, there was a significant difference between the clinical examination results and both the culture and IC smear results in the diagnosis of Microbial Keratitis as P values were significant, and this indicated that both the smear method and the culture methods were better than the clinical examination in the diagnosis of MK (table 2). This agreed with Jain et al., (2007) , Moshtaghion et al., 2020 , and Fanselow et al., 2021 .

Also from the previous results in Tables (2), Acanthamoebic keratitis diagnosis with clinical presentation only is difficult and may be misleading and this is in agreement with (Fanselow et al., 2021) as MK within the first 2 weeks the eye undergo chameleon-like epithelial changes referred to as " dirty epithelium", these changes include a pseudo- dendriform epitheliopathy with grey epithelial opacities which is misdiagnosed as viral keratitis and in late stage may be misdiagnosed as fungal or mixed keratitis. This is in agreement with (Rezaei Kanavi et al., 2013). In this study, an IC smear with Gram stain differentiated the bacteria into Gram positive cocci or bacilli and Gram negative cocci or bacilli, and this was in agreement with Somerville et al. (2021), and this represents a very good guide for the clinician to start treatment.

From the previous results, different correlations have been done in this study between the three diagnostic methods (clinical, IC smear, and culture) of microbial keratitis to detect the accuracy of each diagnostic method using the culture as the gold standard method for microbial keratitis, as shown in tables (2, 3). We detected that there was a significant difference between the diagnosis of MK caused by Acanthamoeba by clinical examination and wet film; there was a significant difference between the diagnosis of both bacterial and fungal keratitis by clinical examination and smear stain methods; but there was a non-significant difference between the diagnosis of MK by smear stain methods and its diagnosis by culture methods. So, it indicates that we can diagnose MK microbiologically at the level of the smear only without waiting for the culture results, which is expensive and time-consuming. Zemba et al., (2022) , Alkatan et al., (2019) , Shabrawy et al., (2013) ,Rai et al., (2016) , Jain et al., (2007) , Shen and Rahman, (2012) , Kim et al., (2006) agreed with our findings.

Conclusion

The impression cytology smear-direct microscopy examination is a simple, quick, non-invasive, non-traumatic, inexpensive, and accurate method for diagnosing microbial keratitis caused by bacteria, fungi, and protozoa. It can differentiate between these three different causes: Culture is the cornerstone for the diagnosis of microbial keratitis; we can consider the smear as a pre-culture diagnostic step in microbiology investigation, which is trustable to start specific medical treatment before culture results are known; clinical examination and risk factors are important guides to direct the diagnosis but not a steady step to start specific antimicrobial treatment.

Conflict of interests

The authors declare that they have no conflict of interests.

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Compliance with Ethics Requirements

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national), ethical approval code 32169\03\18.

Data Availability

This published paper and/or its supporting file includes all the data created or assessed during this study.

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