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ORIGINAL ARTICLE

Polymerase Chain Reaction-Guided Diagnosis of Infective Keratitis – A Hospital Based Study

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ABSTRACT

Purpose: To compare polymerase chain reaction (PCR) to microbial culture and smear for detection and identification of bacterial and fungal pathogens in suspected microbial keratitis.

Materials and methods: Corneal scrapings from 88 patients with suspected infectious keratitis were subjected to routine bacterial culture and sensitivity, Gram's stain, fungal culture; potassium hydroxide (KOH) wet mount, and PCR. PCR was performed with primer pairs targeted to the 16S and 18S r RNA gene. The result of the PCR was compared with conventional culture and Gram staining method.

Results: By broad-range PCR, 40 (45.45%) cases were positive for fungi (90.9% sensitivity), 26 (29.5%) of them were culture positive (59.09% sensitivity), 29 (33%) of all patients were positive for bacteria by broad-range PCR (87.9% sensitivity) and 19 (21.6%) were culture positive (57.58% sensitivity). The time taken for PCR assay was 4–8 h whereas positive fungal cultures took 2–10 days and bacterial culture from 2 to 4 days. Smears were positive for fungi in 29 eyes (33% of cases, 65.91% sensitivity) and for bacteria in 11 eyes (12.5% of cases, 33.33% sensitivity).

Conclusions: DNA amplification with universal primers is a promising diagnostic tool in cases of infectious keratitis where routine laboratory culture failed to identify the pathogen. PCR may be performed in cases where the results of corneal scraping stains are negative without waiting for the results of the culture.

Keywords: Infective keratitis, corneal scrapping, broad-range PCR, universal fungal primers, panbacterial primers

INTRODUCTION

Infective keratitis, especially fungal keratitis, is a serious ocular infection and a major cause worldwide visual loss.[1,2]

An effective treatment depends on rapid detection and identification of the causative agent.

Previous researches revealed that early diagnosis and treatment would lead to better visual outcome.[3] The clinical diagnosis of microbial keratitis often relies on a history of infectious exposure and the morphological features of corneal inflammation.[4] Ophthalmologists use some distinctive though not pathognomonic signs to differentiate bacterial, fungal and amoebic pathogens of the cornea.[5–7]

Laboratory diagnosis of the infective agent in a corneal sample although recommended,[8] may not be regularly obtained due to time, cost and availability.[9] Laboratory diagnosis of infectious keratitis is mainly based on culture of corneal scrapes and direct smear methods.[3] Culture results are highly specific but have suboptimal sensitivity, generally yielding results in fewer than 70% of cases.[10] Cultures also need a long incubation time especially for fungal keratitis. Direct smears also can lead to misdiagnosis.[3,11] The capacity for detection and identification of genomic material in any type of sample has allowed the diagnosis of many genetic or infectious diseases based on the DNA sequence. Molecular diagnosis of ocular infections

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is based on DNA detection of microorganisms by polymerase chain reaction (PCR) in ocular samples.[12] Recently, PCR-based assays have been developed as rapid diagnostic tools for detecting infective keratitis.[13–16]

The main goal of the present study was to compare the positivity and sensitivity of PCR, microbial culture and smear in the diagnosis of Fungal and Bacterial keratitis among Egyptian population.

MATERIALS AND METHODS

Our study was conducted in Cairo University hospitals in the period from December 2008 to March 2011. The research method adhered to the tenets of the Declaration of Helsinki. Eighty-eight patients admitted through the ophthalmic casualty room with corneal ulcers were included in this study. Sixty patients were males, 28 were females. The ages of the patients ranged from 4 to 70 years. Mean age was 42 years. All the patients were examined with a standard written protocol that included detailed history with regards to duration of symptoms, the precipitating factors, trauma, treatment taken and delay in reporting to a medical practitioner, contact lens wear and previous surgeries (intra- and extraocular). Ocular status before the onset of the ulcer and the systemic condition of the patient were noted with special attention to diabetes mellitus and use of systemic or topical steroids.

Clinical Examination

Thorough examination of the involved and fellow eyes was performed. The external ocular examination included lid and adnexal abnormalities such as entropion, trichiasis, skin lesions for vesicle or healed scars to rule out a herpetic cause, dry eye and conditions predisposing to exposure induced keratitis. Careful examination of the involved eye was performed on slit lamp biomicroscope. Pictorial documentation of the ulcer size, site, depth, extent of infiltration, perforation if any, fluorescence (2%) staining, hypopyon if any and scleral involvement were made. Visual acuity and digital intraocular tension were recorded. Ultrasonographic examination of the posterior segment was performed to rule out suspected endophthalmitis. Clinical photographs were recorded in each case and saved for documentation and further follow-up studies.

Obtaining Corneal Scrapings

After the informed consent was obtained from the patients, the involved eye was anesthetized with 0.4% benoxinate eye drops. All sterile surgical precautions were taken to avoid contamination during sample collection. A sterile wire speculum was applied, and the superficial debris and mucus strands were cleaned. The edge of the ulcer was biopsied with a sterile blade 15 and colibri forceps on slit lamp biomicroscope, with care being taken not to perforate the cornea. Two samples from corneal tissue were obtained ranging from 1 to 2 mm in diameter. Each sample was transferred to a sterile Eppindorf tube. They were sent to our Department of Microbiology

Diagnostic Procedures

Corneal scrapings were collected in all cases, and the samples were subjected to Potassium hydroxide (KOH) wet mount, Gram's stain and bacterial culture/sensitivity, and fungal culture. Part of corneal tissue was preserved at –70°C till tested by PCR.

(a) Smear Examination: KOH wet mount and Gram's staining of each smear was made and examined immediately under microscope for the presence of any hyphae. The Gram's-stained slide was also examined microscopically for bacteria and fungal hyphae.

(b) Culture: The collected samples were cultured on blood and MacConkey's agars and on chocolate agar incubated in 5% CO_2 at 35°C, on Sabouraud's dextrose agar incubated at 25°C for up to 21 days and examined every 48 h for any growth. In case of growth, a wet mount was prepared to study the detailed microscopic morphology of the isolate. Part of corneal tissue was put in ten ml trypcase soy broth and incubated at 37°C for 21 days and sub cultured if turbidity was noticed. Microorganisms were identified by their growth characteristics, Gram-stained smears and routine biochemical reactions.

(c) Broad-Range PCR:

(1) NA Extraction: Corneal tissue was crushed homogenized in hypotonic Tris–HCL buffer pH 8.0 (Sigma). Extraction of DNA was performed using the QI Amp DNA mini kit (50) (QIAGEN GmbH, Hilden, Germany; Cat. No.51304).

(2) DNA Amplification:

(i) mplification of D1/D2 region of fungal DNA: Using the universal fungal primers, UNI-F forward primer (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and UNI-R reverse primer (5′-GGTCCGTGTTTCAAGACG-3′),[17] (Oligo, version 3.4; National Biosciences, Inc). Preparation of PCR mix (total volume 50 µL): The master mix contained 100 ng to 1 pg of genomic DNA, 1× PCR buffer and 0.4 µM concentrations of forward and reverse primer pairs. The PCR was performed for 35 cycles in thermal cycler

(Master cycler 5330, Hamburg). The PCR program involved 15 min of initial activation at 95°C, 30 s of denaturing at 95°C, 30 s of annealing at 50°C and 30 s of extension at 72°C, followed by a 7-min final extension at 72°C. An agarose gel electrophoresis was performed to confirm the synthesis of amplicons. PCR products were separated by electrophoresis through 1.8% agarose gel, visualized under UV light and compared at 615 bp of DNA marker[17] (Boehringer Manheim, Germany, 100 bp DNA ladder).

(ii) Amplification of 16S r RNA bacterial gene: We used panbacterial primers: forward primer 63f (5′- CAG GCC TAA CAG ATG CAA GTC-3′) and reverse primer 1387r (5′- GGG CGG WGT GTA CAA GGC-3′), [18] (Oligo, version 3.4; National Biosciences, Inc.). Preparation of PCR mix (total volume 50 µL): For each test sample 10 µL of extracted DNA was added to 0.2 µL Taq polymerase (Promega, USA), 2.5 µL of DNA 1x PCR buffer containing 1.5 mmol mgcl2 and 200 µmol of each dNTP, 20 pmol of each primer, diethyl pyrocarbonate treated water is added to reach a final volume of 50 µL. PCR protocol: Amplification was done in the thermal cycler (Master cycler 5330, Hamburg). The cycler was programmed to perform 30 cycles consisting of 95°C for 1 min, 55°C for 1 min. and 72°C for 1.5 min. followed a final extension step of 5 min. at 72°C.[18] PCR products were separated by electrophoresis through 1.8% agarose gel by standard methods, and they were visualized under UV light and compared at 1324 bp of DNA marker.[18] (Boehringer Manheim, Germany, 100 bp DNA ladder).

RESULTS

Results of Examination for Fungus

Culture Positivity

Of the 88 cases of clinically suspected infectious keratitis studied, 26 (29.5%) were culture positive. Of these 26 positive cases, *Aspergillus* spp. was the predominant isolates (53.8%) followed by *Fusarium* spp., *Penicillium* spp. and *Candida* spp. each (15.4%). Among the *Aspergillus* spp. *A. flavus* was more commonly isolated than *A. fumigatus* (4:1). Smear Positivity: KOH wet mount was found to be positive for fungal hyphae in 29 (33%) of Diagnostic test cases. Of the 26 culture-positive cases, KOH picked up 22 (84.6%). PCR Positivity: PCR results of representative positive and negative samples, along with molecular weight standards are shown in Figure 1. PCR was positive in 40 of 88 cases (45.45%). Of the 26 culture-positive cases, PCR was positive in 22 (84.6%). PCR was positive in 18 of 62 (29%) of culture-negative samples (Tables 1 and 2), 14 (77.8%) of those had received antifungal therapy. Four culture-positive cases were negative by PCR and they grew *Aspergillus* species. The time taken for PCR assay was 4–8 h whereas positive fungal cultures took 2–10 days.

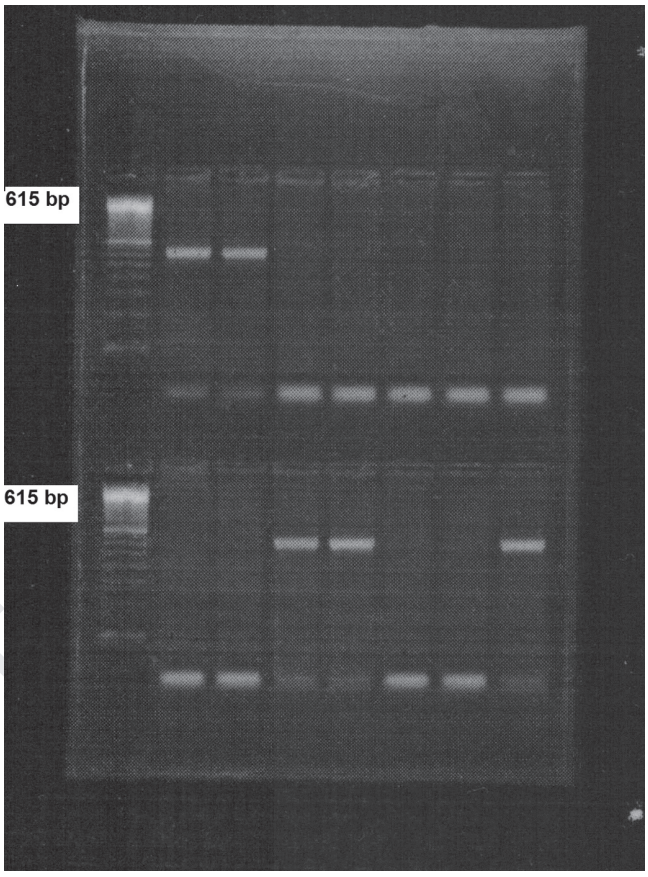

FIGURE 1 Agarose gel electrophoresis of panfungal primer broad-range PCR. Above: Lane 1: DNA marker, positive lanes 2–3 with band 615 bp, negative lanes 4–8. Below: Lane 1: DNA marker, positive lanes 4–5, 8 and negative lanes 2, 3, 6, 7. Molecular weight of the PCR fragments is marked on the left side of the pictures.

TABLE 1 Results of examination for fungus.

Results for fungal pathogens	Culture	KOH	PCR
Positive in all tests	22		
Positive in KOH and culture	0		
Positive in culture and PCR	0		
Positive in KOH and PCR		7	
Positive in each test alone	4	0	11

TABLE 2 Results of culture for fungus and broad-range PCR using the universal fungal primer.

Culture	Broad-range PCR for D1/D2 fungal gene	
	Positive (non = 40)	Negative (non = 44)
Positive (non = 26)	22	4
Negative (non = 62)	18	44

n, number of patients.

Results of Examination for Bacteria

Culture Positivity

Out of the 88 samples of corneal scrapings studied 19 (21.6%) were culture positive. The most frequent bacterial pathogens isolated were *Pseudomonas* species 10/19 (52.6%), followed by *Streptococcus pneumonia* 7/19 (36.8%) and *Klebsiella* species 2/19 (10.5%). Smear Positivity: Of the 19 culture-positive cases, Gram stain was positive in 11 (12.5%) of cases. PCR Positivity: Broad-range PCR analysis of the corneal tissue using the panbacterial primer for amplification of 16S rRNA bacterial gene revealed 29 (33%) positive cases with a band at 1324 bp (Figure 2). Of the 19 samples culture positive for bacteria, 15 (78.9%) were PCR positive, while four samples were culture positive and PCR negative (Tables 3 and 4).The four samples were positive by culture and negative by PCR were culture positive for *Streptococcus pneumoniae*. The PCR positivity in culture-negative samples was 14 of 69 (20.3%), all of them had history of antibiotic intake at the time of sampling. The time taken for PCR assay was 4–8 h whereas positive bacterial culture from 2 to 4 days.

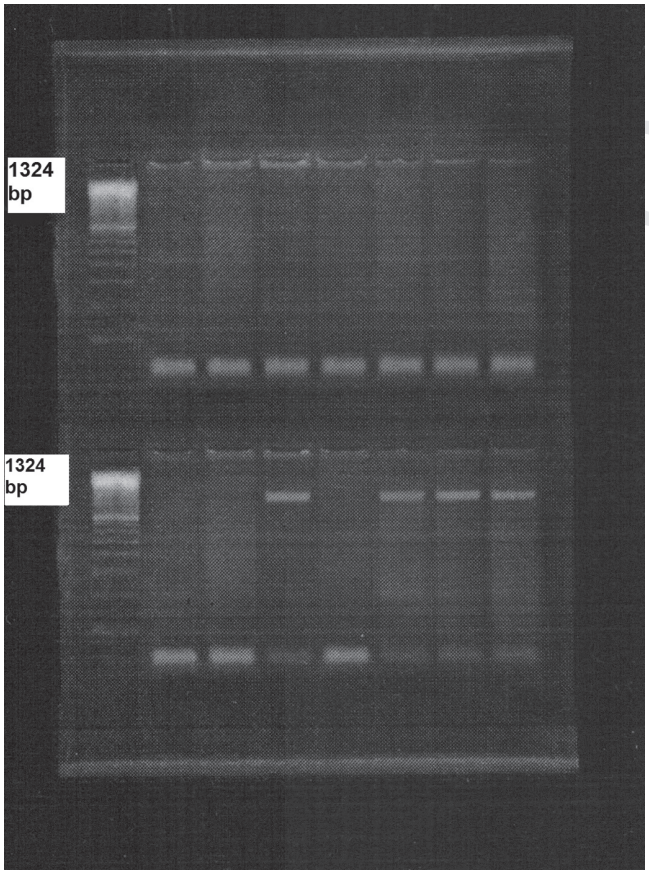

FIGURE 2 Agarose gel electrophoresis of panbacterial primer broad-range PCR. Above: Lane 1: DNA marker, all lanes are negative. Below: Lane 1: DNA marker, positive lanes 4, 6–8 with band at 1324 bp and negative lanes 2, 3, 5. Molecular weight of the PCR fragments is marked on the left side of the pictures.

Fourteen patients (15.9%) with culture negative and PCR negative gave history of contact lens. For calculation of the sensitivity of the tests done (smear, culture and PCR), we considered the microorganism present if it could be detected in any of these tests. We considered the microorganism absent if all the tests were negative. Of 88 cases, 44 (50%) cases were positive for fungus by direct smear, culture and PCR and 33 (38.5%) of cases were positive for bacteria by direct smear, culture and PCR (Table 5).

None of our patients revealed any double positivity for bacterial and fungal pathogens by culture or PCR.

DISCUSSION

In this study, we evaluated the efficacy of the three diagnostic methods, comparing the results of PCR with direct smear and culture of 88 corneal samples. The direct smear done for corneal scrapings is considered by most clinicians as a rapid and sensitive method in the diagnosis of infectious keratitis. Our study revealed that KOH wet mount obtained 33% positivity and sensitivity of 65.9% for fungal hyphae.

Embong et al.,[19] reported a figure of 30% positive results for fungi with KOH. Vengayil et al.,[20] found fungal hyphae in KOH in 40% of cases with sensitivity of 60%. Chowdhary and Singh,[21] recorded sensitivity of 62% with KOH. Bharathi et al.,[22] reported higher sensitivity with KOH wet mount (99.3%) in the detection of fungi. Ferrer and Alió,[23] obtained 66.6% sensitivity with Gram and Calcoflour stains.

In this study, Gram stain for bacteria obtained positivity of 12.5% and sensitivity of 33.3%. Bashir et al.,[24] reported that the sensitivity of Gram stain was 57.14% as compared to culture. Maske et al.,[25] found that Gram stains were positive for organisms in 27% patients. 29.5% were culture positive for fungus (59.09% sensitivity) and 21.6% were culture positive for bacteria (57.58% sensitivity), 72 (81.8%) of our patients had received antifungal therapy and all of them had history

TABLE 3 Results of examination for bacterial pathogens.

Results for bacterial pathogens	Gram	Culture	PCR
Positive in all tests	11		
Positive in Gram and culture	0		
Positive in culture and PCR		4	
Positive in Gram and PCR	0		0
Positive in each test alone	0	4	14

TABLE 4 Results of bacterial culture and broad-range PCR using the panbacterial primer.

Culture	Broad-range PCR for 16 S RNA gene	
	Positive (n = 29)	Negative (n = 61)
Positive (n = 19)	15	4
Negative (n = 69)	14	55

n, number of patients.

TABLE 5 The positivity and sensitivity of the tests done (smear, culture and PCR).

Test	Fungus		Bacteria	
	Positivity (total = 88 eyes)	Sensitivity (95% CI)	Positivity (total = 88 eyes)	Sensitivity (95% CI)
Smear	29 (33%)	65.91% (50.08–79.51%)	11 (12.5%)	33.33% (17.96%–51.83%)
Culture	26 (29.5%)	59.09% (43.25–73.66%)	19 (21.6%)	57.58% (39.22%–74.52%)
PCR	40 (45.45%)	90.91% (78.33–97.47%)	29 (33%)	87.88% (71.80%–96.60%)

of antibiotic intake at the time of sampling. Other authors,[20] reported similar figures of culture positivity (25%) and attributed these low figures to the intake of antifungal therapy

Other authors,[23] reported similar sensitivity of 59.3% by culture for fungus and others,[26] reported higher positivity of 51.9% for fungus and bacteria.

PCR positivity in culture-negative samples was 32 of 43 (74.4%) 18 fungal and 14 bacterial results. Kim et al.,[26] reported 88% PCR positivity in culture-negative samples, 28 fungal and 18 bacterial. The main problem with culture from corneal scraping specimens is the small amount of material that can be obtained for diagnosis, increasing the risk of false-negative results. Additionally, some fungal species cannot be cultured or grow slowly and have fastidious growth requirements.

Other study shows higher sensitivity when a biopsy or cornea is analyzed probably due to some fungi that are found in the deep stroma. This highlights the importance of an adequate sample to get a higher probability of positive results; the depth and amount of the corneal sample should be abundant to increase the microbial load.[23]

This difference between culture and stains may be explained by the fact that the positive result of culture requires viable organisms whereas a stain test can detect both viable and nonviable organisms. In addition, some viable fungal structures in the eye do not grow under laboratory conditions due to the shift of the growth condition (temperature, humidity and substrate).[23] The culture was positive only if the sample contained viable organisms, while a PCR-based test will detect both viable and nonviable organisms. *Aspergillus* spp. was the predominant isolates (53.8%) followed by *Fusarium* spp., *Penicillium* spp. and *Candida* spp. each (15.4%). Other authors isolated *Fusarium* spp. as the predominant fungal pathogen[19,26,27] (Figure 3).

The most frequent bacterial pathogens isolated were *Pseudomonas* species (52.6%), followed by *Streptococcus pneumonia* (36.8%). Other authors,[26] isolated *Corynebacterium* spp. followed by *Streptococcus pneumonia* and *Pseudomonas* species. PCR for fungi was positive in 45.45% of cases (90.9% sensitivity) and was positive in 30.7% of cases for bacterial pathogens (87.9% sensitivity). Embong et al.,[19] reported similar sensitivity in detecting fungi. Other authors reported positive fungal samples ranging from 70 to 97%.[13,20,23,28] They stated that the high percentage of PCR positivity may be because the selected subjects in their study were all with proven fungal keratitis. The data reported by Kim et al.,[26] yield PCR sensitivity of about 86% for bacterial pathogens. Four culture-positive cases for *Aspergillus* spp were negative by PCR and another four culture-positive cases for *Streptococcus pneumonia* were negative by PCR. Embong et al.,[19] reported similar findings and stated that this could be due to insufficient fungal elements present in corneal scraping or due to sequence variation of 18S rRNA gene of this fungus. It is possible that different layers of corneal scraping were used for culture, Gram stain and PCR. Hence, the corneal scraping submitted for PCR may have had insufficient fungal elements relative to the samples submitted for culture and Gram stain. PCR-negative results in culture-positive patients may result from technical factors in some Gram-positive bacteria, as Gram-positive bacteria were not detected as efficiently as the Gram-negative ones in some studies.[29–31] As an example, difficulties in breaking the cell walls of Gram-positive organisms using proteinase K enzyme during sample preparation, resulted in a failure in the DNA extraction process.[30,31] Therefore, using a more effective method of breaking the cell walls such as mechanical disruption of bacteria could be a more optimal solution.[30]

Other organisms known to cause microbial keratitis, such as *Acanthamoeba* or microsporidia, would not be detected by panbacterial and panfungal primer sets.

Limitations of the study: Broad-range PCR should be followed by DNA sequencing for identification of positive cases by PCR to species level to be able to identify the isolated organism as a true pathogen or a possible contaminant and to allow the assessment of concordance between PCR and culture. Moreover, the lack of species identification in corneal infections prevents precise knowledge of antimicrobial therapy efficiency and species epidemiology.

However, in most cases of keratitis, the most important laboratory information that the ophthalmologist needs to know is whether the infectious agent is fungal or bacterial. They often hesitate to initiate antifungal therapy in fungal culture-negative cases due to the risk of drug associated toxicity. Positive PCR results that are available earlier than culture will justify the use of antifungal agents promptly, resulting in improved visual outcome.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

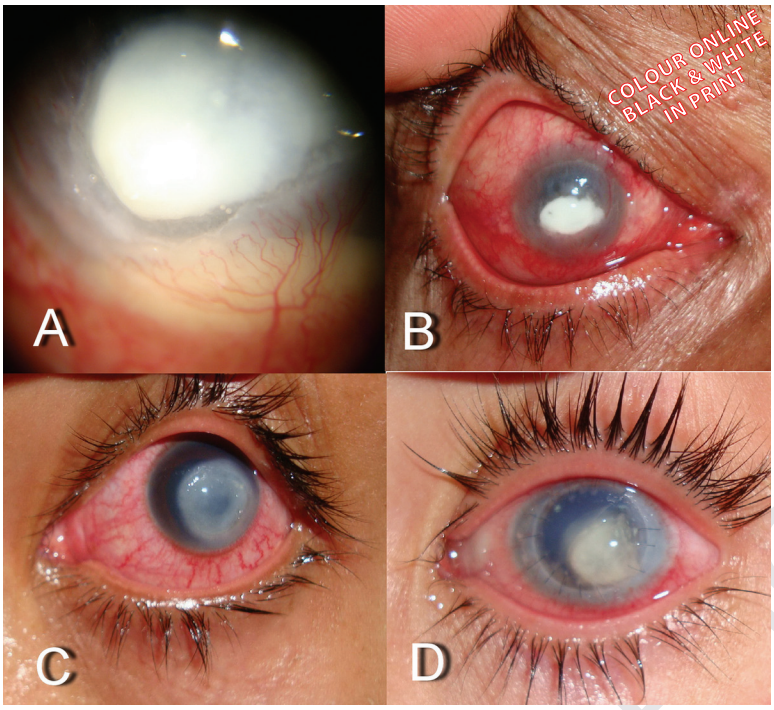

FIGURE 3 PCR proved to be fungal in A, B. Culture yielded *Candida* in A and *Penicillium* in B. PCR proved to be bacterial in C, D. Culture yielded *Pseudomonas* in C and *Streptococcus pneumonia* involving a corneal graft in *D*.

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Polymerase Chain Reaction-Guided Diagnosis of Infective Keratitis – A Hospital Based Study

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ABSTRACT

Purpose: To compare polymerase chain reaction (PCR) to microbial culture and smear for detection and identification of bacterial and fungal pathogens in suspected microbial keratitis.

Materials and methods: Corneal scrapings from 88 patients with suspected infectious keratitis were subjected to routine bacterial culture and sensitivity, Gram's stain, fungal culture; potassium hydroxide (KOH) wet mount, and PCR. PCR was performed with primer pairs targeted to the 16S and 18S r RNA gene. The result of the PCR was compared with conventional culture and Gram staining method.

Results: By broad-range PCR, 40 (45.45%) cases were positive for fungi (90.9% sensitivity), 26 (29.5%) of them were culture positive (59.09% sensitivity), 29 (33%) of all patients were positive for bacteria by broad-range PCR (87.9% sensitivity) and 19 (21.6%) were culture positive (57.58% sensitivity). The time taken for PCR assay was 4–8 h whereas positive fungal cultures took 2–10 days and bacterial culture from 2 to 4 days. Smears were positive for fungi in 29 eyes (33% of cases, 65.91% sensitivity) and for bacteria in 11 eyes (12.5% of cases, 33.33% sensitivity).

Conclusions: DNA amplification with universal primers is a promising diagnostic tool in cases of infectious keratitis where routine laboratory culture failed to identify the pathogen. PCR may be performed in cases where the results of corneal scraping stains are negative without waiting for the results of the culture.

KEYWORDS: Infective keratitis, corneal scrapping, broad-range PCR, universal fungal primers, panbacterial primers

INTRODUCTION

Infective keratitis, especially fungal keratitis, is a serious ocular infection and a major cause worldwide visual loss.[1,2]

An effective treatment depends on rapid detection and identification of the causative agent.

Previous researches revealed that early diagnosis and treatment would lead to better visual outcome.[3] The clinical diagnosis of microbial keratitis often relies on a history of infectious exposure and the morphological features of corneal inflammation.[4] Ophthalmologists use some distinctive though not pathognomonic signs to differentiate bacterial, fungal and amoebic pathogens of the cornea.[5–7]

Laboratory diagnosis of the infective agent in a corneal sample although recommended,[8] may not be regularly obtained due to time, cost and availability.[9] Laboratory

diagnosis of infectious keratitis is mainly based on culture of corneal scrapes and direct smear methods.[3] Culture results are highly specific but have suboptimal sensitivity, generally yielding results in fewer than 70% of cases.[10] Cultures also need a long incubation time especially for fungal keratitis. Direct smears also can lead to misdiagnosis.[3,11] The capacity for detection and identification of genomic material in any type of sample has allowed the diagnosis of many genetic or infectious diseases based on the DNA sequence. Molecular diagnosis of ocular infections is based on DNA detection of microorganisms by polymerase chain reaction (PCR) in ocular samples.[12] Recently, PCR-based assays have been developed as rapid diagnostic tools for detecting infective keratitis.[13–16]

The main goal of the present study was to compare the positivity and sensitivity of PCR, microbial culture and smear in the diagnosis of Fungal and Bacterial keratitis among Egyptian population.

MATERIALS AND METHODS

Our study was conducted in Cairo University hospitals in the period from December 2008 to March 2011. The research method adhered to the tenets of the Declaration of Helsinki. Eighty-eight patients admitted through the ophthalmic casualty room with corneal ulcers were included in this study. Sixty patients were males, 28 were females. The ages of the patients ranged from 4 to 70 years. Mean age was 42 years. All the patients were examined with a standard written protocol that included detailed history with regards to duration of symptoms, the precipitating factors, trauma, treatment taken and delay in reporting to a medical practitioner, contact lens wear and previous surgeries (intra- and extraocular). Ocular status before the onset of the ulcer and the systemic condition of the patient were noted with special attention to diabetes mellitus and use of systemic or topical steroids.

Clinical Examination

Thorough examination of the involved and fellow eyes was performed. The external ocular examination included lid and adnexal abnormalities such as entropion, trichiasis, skin lesions for vesicle or healed scars to rule out a herpetic cause, dry eye and conditions predisposing to exposure induced keratitis. Careful examination of the involved eye was performed on slit lamp biomicroscope. Pictorial documentation of the ulcer size, site, depth, extent of infiltration, perforation if any, fluorescence (2%) staining, hypopyon if any and scleral involvement were made. Visual acuity and digital intraocular tension were recorded. Ultrasonographic examination of the posterior segment was performed to rule out suspected endophthalmitis. Clinical photographs were recorded in each case and saved for documentation and further follow-up studies.

Obtaining Corneal Scrapings

After the informed consent was obtained from the patients, the involved eye was anesthetized with 0.4% benoxinate eye drops. All sterile surgical precautions were taken to avoid contamination during sample collection. A sterile wire speculum was applied, and the superficial debris and mucus strands were cleaned. The edge of the ulcer was biopsied with a sterile blade 15 and colibri forceps on slit lamp biomicroscope, with care being taken not to perforate the cornea. Two samples from corneal tissue were obtained ranging from 1 to 2 mm in diameter. Each sample was transferred to a sterile Eppindorf tube. They were sent to our Department of Microbiology

Diagnostic Procedures

Corneal scrapings were collected in all cases, and the samples were subjected to Potassium hydroxide (KOH) wet mount, Gram's stain and bacterial culture/sensitivity, and fungal culture. Part of corneal tissue was preserved at –70°C till tested by PCR.

(a) Smear Examination: KOH wet mount and Gram's staining of each smear was made and examined immediately under microscope for the presence of any

hyphae. The Gram's-stained slide was also examined microscopically for bacteria and fungal hyphae.

(b) Culture: The collected samples were cultured on blood and MacConkey's agars and on chocolate agar incubated in 5% CO_2 at 35°C, on Sabouraud's dextrose agar incubated at 25°C for up to 21 days and examined every 48 h for any growth. In case of growth, a wet mount was prepared to study the detailed microscopic morphology of the isolate. Part of corneal tissue was put in ten ml trypcase soy broth and incubated at 37°C for 21 days and sub cultured if turbidity was noticed. Microorganisms were identified by their growth characteristics, Gram-stained smears and routine biochemical reactions.

(c) Broad-Range PCR:

(1) DNA Extraction: Corneal tissue was crushed homogenized in hypotonic Tris–HCL buffer pH 8.0 (Sigma). Extraction of DNA was performed using the QI Amp DNA mini kit (50) (QIAGEN GmbH, Hilden, Germany; Cat. No.51304).

(2) DNA Amplification:

(i) Amplification of D1/D2 region of fungal DNA: Using the universal fungal primers, UNI-F forward primer (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and UNI-R reverse primer (5′-GGTCCGTGTTTCAAGACG-3′),[17] (Oligo, version 3.4; National Biosciences, Inc). Preparation of PCR mix (total volume 50 µL): The master mix contained 100 ng to 1 pg of genomic DNA, 1× PCR buffer and 0.4 µM concentrations of forward and reverse primer pairs. The PCR was performed for 35 cycles in thermal cycler (Master cycler 5330, Hamburg). The

PCR program involved 15 min of initial activation at 95°C, 30 s of denaturing at 95°C, 30 s of annealing at 50°C and 30 s of extension at 72°C, followed by a 7-min final extension at 72°C. An agarose gel electrophoresis was performed to confirm the synthesis of amplicons. PCR products were separated by electrophoresis through 1.8% agarose gel, visualized under UV light and compared at 615 bp of DNA marker[17] (Boehringer Manheim, Germany, 100 bp DNA ladder).

(ii) Amplification of 16S r RNA bacterial gene: We used panbacterial primers: forward primer 63f (5′- CAG GCC TAA CAG ATG CAA GTC-3′) and reverse primer 1387r (5′- GGG CGG WGT GTA CAA GGC-3′), [18] (Oligo, version 3.4; National Biosciences, Inc.). Preparation of PCR mix (total volume 50 µL): For each test sample 10 µL of extracted DNA was added to 0.2 µL Taq polymerase (Promega, USA), 2.5 µL of DNA 1x PCR buffer containing 1.5 mmol mgcl2 and 200 µmol of each dNTP, 20 pmol of each primer, diethyl pyrocarbonate treated water is added to reach a final volume of 50 µL. PCR protocol*:* Amplification was done in the thermal cycler (Master cycler 5330, Hamburg). The cycler was programmed to perform 30 cycles consisting of 95°C for 1 min, 55°C for 1 min. and 72°C for 1.5 min. followed a final extension step of 5 min. at 72°C.[18] PCR products were separated by electrophoresis through 1.8% agarose gel by standard methods, and they were visualized under UV light and

compared at 1324 bp of DNA marker.[T8] (Boehringer Manheim, Germany, 100 bp DNA ladder).

RESULTS

Results of Examination for Fungus

Culture Positivity

Of the 88 cases of clinically suspected infectious keratitis studied, 26 (29.5%) were culture positive. Of these 26 positive cases, *Aspergillus* spp. was the predominant isolates (53.8%) followed by *Fusarium* spp., *Penicillium* spp. and *Candida* spp. each (15.4%). Among the *Aspergillus* spp. *A. flavus* was more commonly isolated than *A. fumigatus* (4:1). Smear Positivity: KOH wet mount was found to be positive for fungal hyphae in 29 (33%) of Diagnostic test cases. Of the 26 culture-positive cases, KOH picked up 22 (84.6%). PCR Positivity: PCR results of representative positive and negative samples, along with molecular weight standards are shown in Figure 1. PCR was positive in 40 of 88 cases (45.45%). Of the 26 culture-positive cases, PCR was positive in 22 (84.6%). PCR was positive in 18 of 62 (29%) of culture-negative samples (Tables 1 and 2), 14 (77.8%) of those had received antifungal therapy. Four culture-positive cases were negative by PCR and they grew *Aspergillus* species. The time taken for PCR assay was 4–8 h whereas positive fungal cultures took 2–10 days.

Results of Examination for Bacteria

Culture Positivity

Out of the 88 samples of corneal scrapings studied 19 (21.6%) were culture positive. The most frequent bacterial pathogens isolated were *Pseudomonas* species 10/19 (52.6%), followed by *Streptococcus pneumonia* 7/19 (36.8%) and *Klebsiella* species 2/19 (10.5%). Smear Positivity: Of the 19 culture-positive cases, Gram stain was positive in 11 (12.5%) of

cases. PCR Positivity: Broad-range PCR analysis of the corneal tissue using the panbacterial primer for amplification of 16S rRNA bacterial gene revealed 29 (33%) positive cases with a band at 1324 bp (Figure 2). Of the 19 samples culture positive for bacteria, 15 (78.9%) were PCR positive, while four samples were culture positive and PCR negative (Tables 3 and 4).The four samples were positive by culture and negative by PCR were culture positive for *Streptococcus pneumoniae*. The PCR positivity in culture-negative samples was 14 of 69 (20.3%), all of them had history of antibiotic intake at the time of sampling. The time taken for PCR assay was 4–8 h whereas positive bacterial culture from 2 to 4 days. Fourteen patients (15.9%) with culture negative and PCR negative gave history of contact lens. For calculation of the sensitivity of the tests done (smear, culture and PCR), we considered the microorganism present if it could be detected in any of these tests. We considered the microorganism absent if all the tests were negative. Of 88 cases, 44 (50%) cases were positive for fungus by direct smear, culture and PCR and 33 (38.5%) of cases were positive for bacteria by direct smear, culture and PCR (Table 5).

None of our patients revealed any double positivity for bacterial and fungal pathogens by culture or PCR.

DISCUSSION

In this study, we evaluated the efficacy of the three diagnostic methods, comparing the results of PCR with direct smear and culture of 88 corneal samples. The direct smear done for corneal scrapings is considered by most clinicians as a rapid and sensitive method in the diagnosis of infectious keratitis. Our study revealed that KOH wet mount obtained 33% positivity and sensitivity of 65.9% for fungal hyphae.

Embong et al.,[19] reported a figure of 30% positive results for fungi with KOH. Vengayil et al.,[20] found fungal hyphae in KOH in 40% of cases with sensitivity of 60%.

Chowdhary and Singh,[21] recorded sensitivity of 62% with KOH. Bharathi et al.,[22] reported higher sensitivity with KOH wet mount (99.3%) in the detection of fungi. Ferrer and Alió,[23] obtained 66.6% sensitivity with Gram and Calcoflour stains.

In this study, Gram stain for bacteria obtained positivity of 12.5% and sensitivity of 33.3%. Bashir et al.,[24] reported that the sensitivity of Gram stain was 57.14% as compared to culture. Maske et al.,[25] found that Gram stains were positive for organisms in 27% patients. 29.5% were culture positive for fungus (59.09% sensitivity) and 21.6% were culture positive for bacteria (57.58% sensitivity), 72 (81.8%) of our patients had received antifungal therapy and all of them had history of antibiotic intake at the time of sampling. Other authors,[20] reported similar figures of culture positivity (25%) and attributed these low figures to the intake of antifungal therapy

Other authors,[23] reported similar sensitivity of 59.3% by culture for fungus and others,[26] reported higher positivity of 51.9% for fungus and bacteria.

PCR positivity in culture-negative samples was 32 of 43 (74.4%) 18 fungal and 14 bacterial results. Kim et al.,[26] reported 88% PCR positivity in culture-negative samples, 28 fungal and 18 bacterial. The main problem with culture from corneal scraping specimens is the small amount of material that can be obtained for diagnosis, increasing the risk of false-negative results. Additionally, some fungal species cannot be cultured or grow slowly and have fastidious growth requirements.

Other study shows higher sensitivity when a biopsy or cornea is analyzed probably due to some fungi that are found in the deep stroma. This highlights the importance of an adequate sample to get a higher probability of positive results; the depth and amount of the corneal sample should be abundant to increase the microbial load.[23]

This difference between culture and stains may be explained by the fact that the positive result of culture requires viable organisms whereas a stain test can detect both viable

and nonviable organisms. In addition, some viable fungal structures in the eye do not grow under laboratory conditions due to the shift of the growth condition (temperature, humidity and substrate).[23] The culture was positive only if the sample contained viable organisms, while a PCR-based test will detect both viable and nonviable organisms. *Aspergillus* spp. was the predominant isolates (53.8%) followed by *Fusarium* spp., *Penicillium* spp. and *Candida* spp. each (15.4%). Other authors isolated *Fusarium* spp. as the predominant fungal pathogen[19,26,27] (Figure 3).[AU: Please check and approve the insertion of the citation for Figure 3 here.]

The most frequent bacterial pathogens isolated were *Pseudomonas* species (52.6%), followed by *Streptococcus pneumonia* (36.8%). Other authors,[26] isolated *Corynebacterium* spp. followed by *Streptococcus pneumonia* and *Pseudomonas* species. PCR for fungi was positive in 45.45% of cases (90.9% sensitivity) and was positive in 30.7% of cases for bacterial pathogens (87.9% sensitivity). Embong et al.,[19] reported similar sensitivity in detecting fungi. Other authors reported positive fungal samples ranging from 70 to 97%.[13,20,23,28] They stated that the high percentage of PCR positivity may be because the selected subjects in their study were all with proven fungal keratitis. The data reported by Kim et al.,[26] yield PCR sensitivity of about 86% for bacterial pathogens. Four culture-positive cases for *Aspergillus* spp were negative by PCR and another four culture-positive cases for *Streptococcus pneumonia* were negative by PCR. Embong et al.,[19] reported similar findings and stated that this could be due to insufficient fungal elements present in corneal scraping or due to sequence variation of 18S rRNA gene of this fungus. It is possible that different layers of corneal scraping were used for culture, Gram stain and PCR. Hence, the corneal scraping submitted for PCR may have had insufficient fungal elements relative to the samples submitted for culture and Gram stain. PCR-negative results in culture-positive patients may result from technical factors in some Gram-positive bacteria, as Gram-positive

bacteria were not detected as efficiently as the Gram-negative ones in some studies.[29–31] As an example, difficulties in breaking the cell walls of Gram-positive organisms using proteinase K enzyme during sample preparation, resulted in a failure in the DNA extraction process.[30,31] Therefore, using a more effective method of breaking the cell walls such as mechanical disruption of bacteria could be a more optimal solution.[30]

Other organisms known to cause microbial keratitis, such as *Acanthamoeba* or microsporidia, would not be detected by panbacterial and panfungal primer sets.

Limitations of the study: Broad-range PCR should be followed by DNA sequencing for identification of positive cases by PCR to species level to be able to identify the isolated organism as a true pathogen or a possible contaminant and to allow the assessment of concordance between PCR and culture. Moreover, the lack of species identification in corneal infections prevents precise knowledge of antimicrobial therapy efficiency and species epidemiology.

However, in most cases of keratitis, the most important laboratory information that the ophthalmologist needs to know is whether the infectious agent is fungal or bacterial. They often hesitate to initiate antifungal therapy in fungal culture-negative cases due to the risk of drug associated toxicity. Positive PCR results that are available earlier than culture will justify the use of antifungal agents promptly, resulting in improved visual outcome.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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TABLE 1 Results of examination for fungus.[AU: Please check and approve the alignment of Table 1.]

Results for fungal pathogens	Culture	KOH	PCR
Positive in all tests	22		
Positive in KOH and culture	0		
Positive in culture and PCR	0		
Positive in KOH and PCR		7	
Positive in each test alone	4	0	11

TABLE 2 Results of culture for fungus and broad-range PCR using the universal fungal primer.

Culture	Broad-range PCR for D1/D2 fungal gene	
	Positive (non = 40)	Negative (non = 44)
Positive (non = 26)	22	4
Negative (non = 62)	18	44

n, number of patients.

TABLE 3 Results of examination for bacterial pathogens.

Results for bacterial pathogens	Gram	Culture	PCR
Positive in all tests	11		
Positive in Gram and culture	0		
Positive in culture and PCR		4	
Positive in Gram and PCR	0		0
Positive in each test alone	0	4	14

TABLE 4 Results of bacterial culture and broad-range PCR using the panbacterial primer.

Culture	Broad-range PCR for 16 S RNA gene	
	Positive (n = 29)	Negative (n = 61)
Positive (n = 19)	15	4
Negative (n = 69)	14	55

n, number of patients.

TABLE 5 The positivity and sensitivity of the tests done (smear, culture and PCR).

Test	Fungus	Bacteria

	Positivity (total = 88 eyes)	Sensitivity (95% CI)	Positivity (total = 88 eyes)	Sensitivity (95% CI)
Smear	29 (33%)	65.91% (50.08–79.51%)	11 (12.5%)	33.33% (17.96%–51.83%)
Culture	26 (29.5%)	59.09% (43.25–73.66%)	19 (21.6%)	57.58% (39.22%–74.52%)
PCR	40 (45.45%)	90.91% (78.33–97.47%)	29 (33%)	87.88% (71.80%–96.60%)

FIGURE 1 Agarose gel electrophoresis of panfungal primer broad-range PCR. Above: Lane 1: DNA marker, positive lanes 2–3 with band 615 bp, negative lanes 4–8. Below: Lane 1: DNA marker, positive lanes 4–5, 8 and negative lanes 2, 3, 6, 7. Molecular weight of the PCR fragments is marked on the left side of the pictures.

FIGURE 2 Agarose gel electrophoresis of panbacterial primer broad-range PCR. Above: Lane 1: DNA marker, all lanes are negative. Below: Lane 1: DNA marker, positive lanes 4, 6–8 with band at 1324 bp and negative lanes 2, 3, 5. Molecular weight of the PCR fragments is marked on the left side of the pictures.

FIGURE 3 PCR proved to be fungal in A, B. Culture yielded *Candida* in A and *Penicillium* in B. PCR proved to be bacterial in C, D. Culture yielded *Pseudomonas* in C and *Streptococcus pneumonia* involving a corneal graft in *D*.