

Diagnosis of Microbial Keratitis Causatives by Polymerase Chain Reaction

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Abstract

In order to evaluate the efficiency of using Polymerase Chain Reaction (PCR) in the identifications of microorganisms causing microbial keratitis, 20 corneal scraping samples were collected from patients who attended the Eye Casualty Unit at the Southampton General Hospital in the United Kingdom. Samples cultured on blood agar and chocolate agar incubated at 37 °C for 24hrs and on sabrouad agar at 28 °C for one week. PCR procedure was performed with the primer paired that targeted to the 16S rRNA for bacterial species and 18S rRNA gene for fungal species, in addition to the species specific primer for the most common microbial keratitis causatives microorganisms. Results in the regards showed that out of the 20 presumed cases of keratitis, PCR showed positivity in 75% of them, from these 55% were due to the fungal infection and 20% of the cases indicated that the keratitis belonged to bacterial infections: In comparison, only 25% of positivity was obtained by the cultural method. The species specific primer showed that half of the 20% bacterial infection cases were caused by *S. aureus* and the other 10% referred to *S.epidermidis* infection. While the *candida albicans* primer gave a positive result only in 72% of the original percentage (55%), the rest 28% may belong to the other fungal infection. Depending on the above results, it can be concluded that PCR not only proved to be an effective rapid method for the diagnosis of bacterial and fungal keratitis, but was also more accurate and sensitive method than the culture methods.

Keywords: Bacterial keratitis, polymerase chain reaction, fungal keratitis, corneal ulcer.

Introduction

Microbial keratitis is a serious ocular infection that can cause corneal scarring and opacification. The principal causes of microbial keratitis are bacteria and fungi [1]. The correct and early identification of the etiological agent by analysis of sample is important for the institution of an early and effective antibiotic therapy, and for the prevention of the inappropriate use of broad-spectrum antibiotics by minimizing the potential emergence of resistant bacterial strains [2].

Conventional diagnosis of a bacterial infection mainly relies on culture-based testing. These cultivations usually yield diagnostic results in days or in some cases up to a week after sampling. Furthermore, cultivation of bacteria is not always successful under laboratory conditions. Such failures may occur due to unsuitable culturing conditions and methods for the bacterial species in question. Alternatively, the patient under investigation may have received antimicrobial therapy before sampling [3].

Molecular methods based on nucleic acid amplification and hybridization aim to circumvent these problems and hasten diagnostic procedures. In such methods, the pathogen is simultaneously detected and identified, which results in more rapid diagnoses than those obtained by conventional culturing methods and obviates the need for additional culture tests. Rapid diagnostics can also reduce the use of antimicrobial agents in addition to allowing a faster switch to the most optimum treatment, thus reducing both side-effects and costs [4].

Using PCR techniques for the identification of the pathogens causing bacterial eye disease presents a challenge, given the large number of bacterial pathogens that are commonly encountered. The 16S subunit, or small subunit, of rRNA has been the target of PCR for the identification of bacterial pathogens in systemic diseases. The 16S rRNA contains regions of highly conserved sequences that are common among all previously studied bacteria interspersed with highly variable or divergent sequences that can differentiate one species from another.

Primers that are complementary to conserved sequences of the gene and that flank variable regions can be used to amplify a portion of rRNA or its complementary ribosomal DNA (rDNA). The PCR product can then be sequenced to provide a unique identifier for the bacteria present in the specimen [5].

PCR has been shown to be useful for the culture-independent diagnosis of various microbial infections, including mycosis. A few cases of mycotic keratitis have successfully been diagnosed by PCR. Ribosomal DNA is the most conserved region in the genome, with capabilities of phylogenetic divergence. The whole rRNA gene contains a small subunit (SSU) 18S rRNA, 5.8S rRNA, and a large subunit (LSU) 28S rRNA. Internal transcribed spacer (ITS) region I (ITSI) and ITSII are more variable than the rest of the ribosomal gene subunits, and are found between SSU rRNA and 5.8S rRNA and between 5.8S rRNA and LSU rRNA, respectively. By using this method, useful vision could be restored due to prompt diagnosis and specific antifungal therapy [6].

According to the above and because the very limited studies in Iraq about using genetic methods in the identification of the microbial keratitis causatives agent this study was conducted to evaluate the ability of using PCR in diagnosis of microbial keratitis and compare the results with that obtained from conventional methods.

Materials and Methods

Sampling

Samples for genetic analysis were taken from patients seen in the eye Casualty unit of the Southampton general hospital in Southampton, UK for a period of 5 months from April to September (2011).

All samples were collected by medically qualified personnel, upon completion of the ocular examination and after instillation of topical anesthetic. A sterile syringe needle was used to scrape the area of infection, then the scrapings were divided into two parts; the first (for bacterial isolates) was inoculated onto each of blood agar and chocolate agar and incubated at 37°C for 2 days; and for fungi it was inoculated onto sabouraud agar and incubated at 28 °C for 7 days. After incubation, positive cultures were further analyzed by

subjecting their colonies to some biochemical tests until more specific species were identified. The second part of the corneal scraping was placed in transport buffer (TE buffer) and stored at -20°C until used for further analysis.

Identification of isolates:

Bacterial isolates were identified using standard microbiological methods. After isolation by culture, the API biochemical identification system was used for identification. The fungal isolates were identified according to their morphological characteristics.

RNA extraction and preparation of cDNA:

The procedure for RNA extraction was made according to the kit supplier (Qiagen, Southampton, UK), in order to do the RT-PCR method, the extracted RNA should be converted to cDNA by using the whole transcriptome amplification kit from (Qiagen, Southampton, UK).

PCR amplification:

Primers

The 16s rRNA gene and 18s rRNA were used as the target gene for this study. Also the species specific primers for the most common ocular pathogens were used. The sequences of all primers used in this study are given in Table (1).

Optimal PCR condition:

In order to diagnose the microorganisms that cause microbial keratitis, polymerase chain reaction was used as a diagnostic techniques. The extracted DNA was subjected to amplification with a T-3000 thermocycler (Biometra, Germany) and with the use of primers listed in Table (1). All primers were made by Invitrogen, Southampton, United Kingdom. They were used in 4 sets of PCR reactions as follow:

The first set was standardized by using the universal bacterial primer (27f, 1525r). The 25µl reaction mixture was containing; 12.5µl of go taq green master mix, 1µM of each of upstream and downstream primer, 5µl of template DNA sample and nuclease free water to complete the volume to 25µl. The reaction involved 20 cycles in a series of denaturation at 94 °C for 60 sec, annealing at 50 °C for

30 sec, and extension at 72 °C for 2 minute. There was also a final 5-minute extension step at 72°C.

The second set of PCRs was done by using the universal fungi primer (F, R). The reaction mixture was the same as that used in the first set of PCR reaction, and the reaction conditions involved 50 cycle, after an initial denaturation at 95°C for 5 minutes. Each cycle consisted of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute, and extension at 72°C for 5 minutes.

The third set of PCR reaction was performed by using species specific primer

(*P. aeruginosa*, *S. aureus*, *S. epidermidis*, *S. pneumoniae*) which applied for the samples gave a positive result in the first set of PCR reactions. The reaction mixture was of the same composition as described above but conditions of each set of primer were different as follow, respectively; after an initial denaturation for 2 minute at 95°C, 25 cycles were completed, each consisting of 20 sec at 94°C, 20 sec at 58°C, and 40 sec at 72°C. A final extension of 1 minute at 72°C was applied; for 35 cycles consisting of 94°C for 15 sec, 54°C for 1 min, and 72°C for 2 min.

Table (1)
Oligonucleotide primers used in this study.

Primer name	Primer sequence	Uses	Product size	References
CABF59 F	5-TTGAACATCTCCAGTTTCAAAGGT-3	<i>C.albicans</i>	665	[7]
CADBR125R	5-AGCTAAATTCATAGCAGAAAGC-3			
(27f)	5'-AGAGTTTGATCCTGGCTCAG-3	Universal bacterial	1,500	[8]
(1525r)	5'-AAGGAGGTGATCCARCC-3			
PA-GS-F	GACGGGTGAGTAATGCCTA	<i>Pseudomonas</i> species	618	[9]
PA-GS-R	CACTGGTGTTTCCTTCTATA			
PA-SS-F	GGGGGATCTTCGGACCTCA	<i>P. aeruginosa</i>	956	
PA-SS-R	TCCTTAGAGTGCCACCCG			
F	GTG AAA TTG TTG AAA GGG AA	Universal fungi 18SrRNA	260	[10]
R	GAC TCC TTG GTC CGT GTT			
S4F	GACA ACTAGAGATAGAGCCTTCC	<i>S. aureus</i>	324	[11]
S4R	AGTCGAGTTGCAGACTAC			
91E-F	GGA ATT CAA ATG AAT TGA CGG GGG C	16S rRNAs. <i>epidremidis</i>	478	[12]
13B-R	CGG GAT CCC AGG CCC GGG AAC GTA TTC AC			
9Y	CGGGATCCGGCAAATAAAGCAGTAAATGACTTT	<i>Streptococcus pneumonia</i>	1418	[12]
9Z	GACGGAGCTCGACTAGTCATTTTCTACCTTATC			

then by a final extension step at 72°C for 7 min; an initial denaturation at 94°C for 2 min, followed by 30 cycles of amplification (denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min), and ending with a final extension at 72°C for 5 min. The reaction conditions for *streptococcus* sp primer involved.

The last set of PCR reaction involved primers specific for *C. albicans* to identify samples gave positive results in the second set of PCR reaction. The reaction mixture contained all the components at the same concentration described above, and the reaction conditions involved preheating at 96°C for 2 minute; then 30 cycles of 96 °C for 30 sec, 57 °C for 3 sec and 74 °C for 60 sec.

For all PCR protocols, a reaction mixture without sample DNA was used as a negative control, in addition to using DNA from the most common cause of microbial keratitis as a positive control. The amplified product was then electrophorised in 1% of a garose gel then stained with 0.5 µg/mL ethidium bromide and visualized in an ultraviolet transilluminator.

Results and Discussion

Isolation and identification of microbial keratitis causatives by cultural method:

The 20 specimens were collected from patients seen in the Eye Casualty Unite of Southampton General Hospital in United Kingdom. After culturing on blood agar, chocolate agar and sabaroud agar, the isolates were further identified according to the API biochemical identification system. The fungal isolates were identified according to their morphological characteristics.

This method gave a positivity of about 25%; 20% as bacterial infection and 5% as fungal represented by *C. albicans*. The isolated bacteria were identified as *S. aureus* which was represented by about 10% and *S. epidermidis* by about 10%.

Isolation and identification of microbial keratitis causatives by genetic methods:

Despite that PCR technique is characterized by not requiring a large quantity of DNA sample, concentration of cDNA and RNA were determined by using nanodrope spectrophotometer. Results showed that the recorded range of cDNA concentration was

(2576.95-4147.50) ng/µl, and that of RNA concentration (1.55-24) ng/µl. Such concentrations are considered to be encouraging. In this regard, Embong [13] mentioned that the amount of DNA used for PCR amplification to detect the microbial keratitis was ranged from (10 ng) to (1 fg).

In order to examine the efficiency of PCR technique for fasten and accurate diagnosis of microorganisms causing microbial keratitis, PCR was performed in two steps; the first is by using the universal primers (fungal and bacterial), and the second by using species specific primer for identifying the most common microorganisms causing keratitis to the species level and aiding the ophthalmologist in treatment.

When the specificity of the selected primers was investigated, each primer was exactly specific to the purpose of selection. In most cases of keratitis, most of the important laboratory information needed by the ophthalmologists 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. For this reason, as mentioned previously PCR method involved the using of 4 sets; in the first, the universal bacterial primer (27F, 1525R) was used to amplify cDNA of keratitic patient. As shown in Fig.(1), 4 (20%) of the 20 specimens gave a clear band with a size of (1500 bp) which reveal that these 4 specimens taken from patients an infected with a type of bacterial keratitis.

The second set of PCRs was done by using the universal fungi primer (F, R). Result indicate that 11(55%) of the 20 samples gave a band of (260 bp) in a size as shown in Fig.(2). this means that the specimens were referred to patients infected with mycotic type of keratitis.

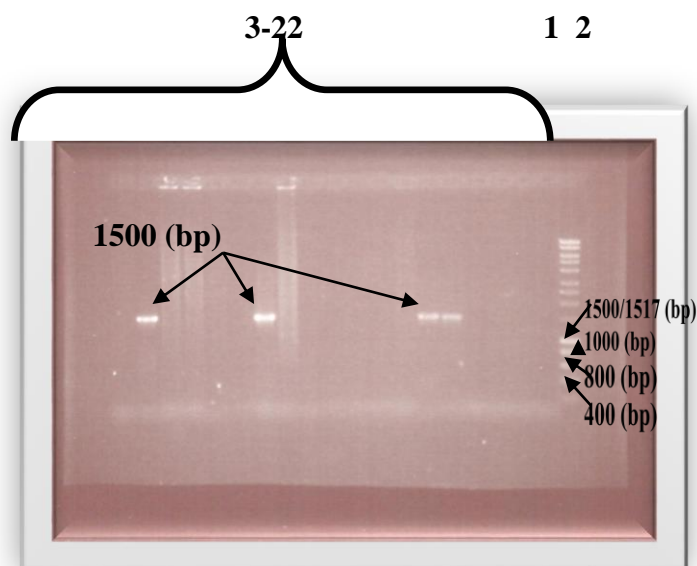


Fig. (1) Gel electrophoresis of PCR product using cDNA extracted from the ocular sample with universal bacterial primer (27F, 1525R). Electrophoresis was performed on 1% agarose gel and run with 100 volt for 60-90 minutes. the lanes:

3-22: cDNA from keratitis patients amplified with universal bacterial primer
1: hyperladder I DNA marker
2: negative control.

In the third set of PCR, the ocular samples, which gave a positive result by using universal bacterial primer, were amplified by using the species specific primers (PA-SS-F, PA-SS-R), (S4F,S4R), (91E-F, 13BR), and (9Y,9Z) for *P. aeruginosa*, *S. aureus*, *S. epidermidis*, *S. pneumonie*, respectively, to detect microorganisms causing the infection to the species level. Results shown in Fig.(3) indicate that the banding patterns were observed in lane number 2 (cDNA amplified with *S. epidermidis* species specific primers 91EF, 13BR), with a molecular weights are (478bp) for each, this mean that the microorganism which caused the infection was belonged to *S. epidermidis* keratitis. Also, it indicates that there were bands in lane number 3 (cDNA amplified with primer S4F, S4R), which referred to the presence of a single band with a molecular weight of (324bp); this reveals that the microorganism which caused the infection was belonged to *S. aureus* keratitis. Finally, lane numbers 4,5

showed no bands when the cDNA was amplified with (PA-SS-F, PA-SS-R) (9Y, 9Z) primers for *P. aeruginosa* and *S. pneumonie* respectively.

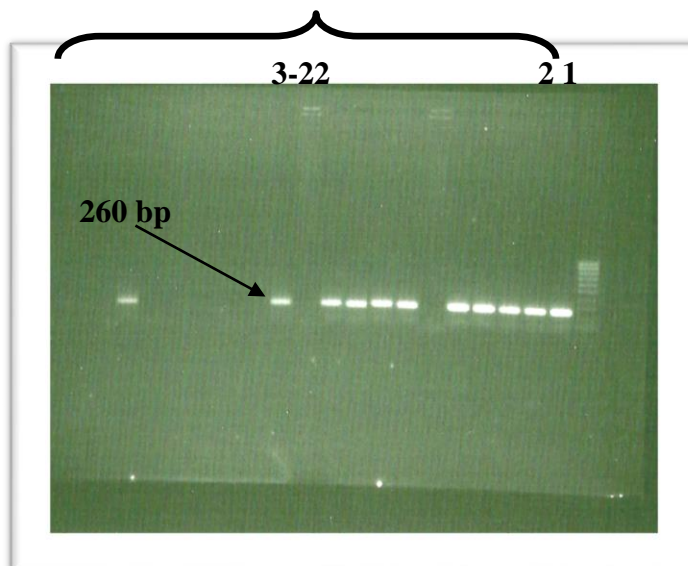


Fig. (2) Gel electrophoresis of PCR product using cDNA extracted from the ocular samples with universal fungal primer (F, R). Electrophoresis was performed on 2% agarose gel and run with 100 volt for 60-90 minutes. The lanes:

1: negative control
2: hyperladder IV DNA marker
3-22: cDNA from keratitis patients amplified with universal fungal primer.

The specimens gave a positive result in the second set of the PCR method (using the universal fungal primer) when has been amplified by the *C. albicans* species specific primer. Results in Fig.(4) showed that 7 (63.63%) of 11 specimens gave a band with a molecular weight (665bp), while no band appeared in the others 4 specimens, which means that it may belong to a species other than *C. albicans*.

Possibility of using the PCR amplification method for the identifications of microorganisms that cause microbial keratitis has been investigated. These results were compared to those obtained by standard cultural methods, PCR method gave positive results of 75% while only 25% was recorded by the cultural method, ensuring that the PCR amplification can be reliably used for detection of pathogens in patients with microbial keratitis. High percentage of positive results

by genetic method might be because that the patients where the specimens taken from were initiated an antibiotic treatment which makes recovery of the organisms for identification more difficult. This came in accordance with Marangon *et al.* [14] who reported that 56% of patients referred to their center were already on topical antibiotic therapy before culture specimens were obtained.

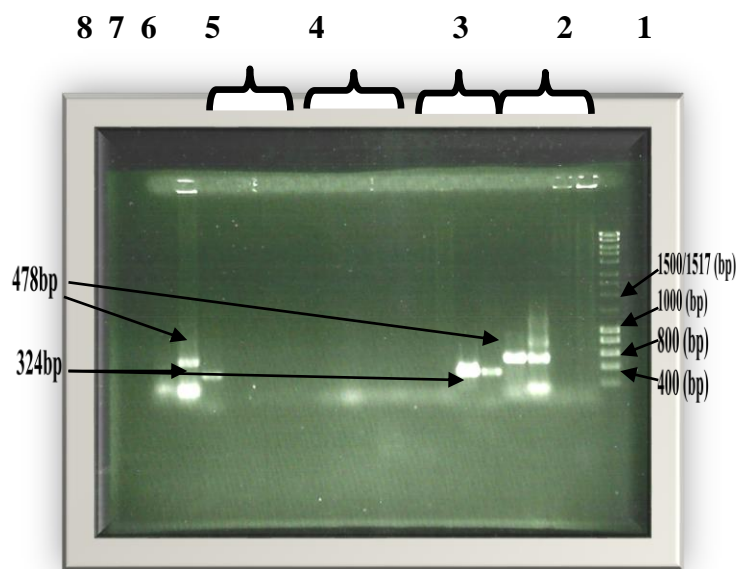


Fig. (3) Gel electrophoresis of PCR product using cDNA extracted from the ocular sample gave positive results in the first set of PCR process with bacterial species specific primer (PA-SS-F, PA-SS-R), (S4F,S4R), (91E-F, 13BR), (9Y,9Z)for *P. aeruginosa*, *S. aureus*, *S. epidermidis*, *S. pneumonie* respectively). Electrophoresis was performed on (1%) agarose gel and run with 100 volt for 60-90 minutes. the lanes:

- 1:** hyperladder IV DNA marker. **2:** cDNA from keratitis patients amplified with *S. epidermidis* species specific primer. **3:** cDNA from keratitis patients amplified with *S. aureus* species specific primer. **4:** cDNA from keratitis patients amplified with *P. aeruginosa* species specific primer. **5:** cDNA from keratitis patients amplified with *S. pneumonie* species specific primer. **6:** positive control of *S. aureus*. **7:** positive control of *S. epidermidis*. **8:** negative control.

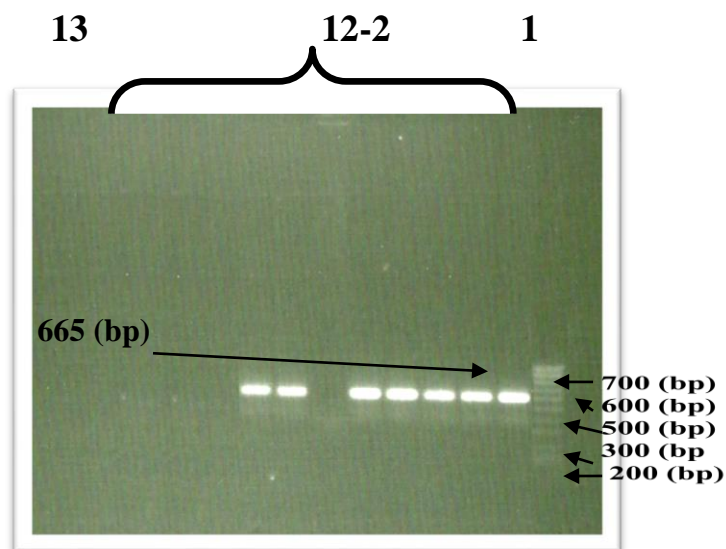


Fig. (4) Gel electrophoresis of PCR product using cDNA extracted from the ocular sample gave positive results in the second set of PCR process with *C. albicans* species specific primer (CABF59F, CADBR125R). Electrophoresis was performed on (1%) agarose gel and run with 100 volt for 60-90 minutes. the lanes:

- 1:** hyperladder IV DNA marker. **2-12:** cDNA from keratitis patients amplified with *C. albicans* species specific primer **13:** negative control.

A 25% of negative results by PCR may be explained by the fact that the specimens for PCR were taken after the patients were treated. The specimens may; therefore, have been inadequate, containing insufficient numbers of organisms for assay detection [15].

The PCR results were close to those of Vengayil *et al.*, [10] who found that the positivity of PCR assays in the presumed cases of fungal corneal ulcers were reached 70%. They also mentioned that the standard techniques for culture in fungal infections are complicated due to some factors such as, slow growing of fungi, unfreshly prepared media, high chance of growth contaminants and the time involved in confirming the culture growth. The culture is positive only if the specimens contain viable organism while a PCR based test detect both viable and non viable organisms.

Gaudio *et al.* [16] on comparing PCR and culture method in 30 presumed cases of fresh and untreated fungal corneal ulcers, positivity of the PCR method was 50%, which is closed to this study. Kalamurthy *et al.* [17] reported that PCR was more sensitive as a diagnostic aid for mycotic keratitis, when fungal DNA was detected in 51% of the 65 specimens tested, whereas the culture method was positive in 43.1% of the samples. Thus the universal fungal primer used was suitable for amplification of fungus specific DNA fragments in the corneal scrapes. Based on the results of this study and the results of the other workers, it may be concluded that PCR can be recommended as a rapid and sensitive diagnostic technique for mycotic keratitis.

In a study by Ferrer and Alio [1], PCR was efficient in detecting very high percentage (92.6%) of all the samples that were positive by the conventional method used. Stain method, for example, 66.7% of positive results, culture method 59.3%, however, PCR detected all samples which were positive in the other both method (together). For fungi the time taken by PCR assay was (4 to 8) hrs whereas positive fungal cultures took 1 to 8 days. Identification at species level by the molecular methods was possible in all cases. PCR not only proved to be an effective rapid method for the diagnosis of fungal keratitis, but was also more sensitive than the stain and culture methods. They mentioned also that the fungal PCR must be added as the screening diagnosis test when an early mycotic keratitis is suspected, and they describe the molecular identification as the gold standard technique for the identification of corneal fungal pathogens.

Conclusion

PCR not only proved to be an effective rapid method for the diagnosis of fungal and bacterial keratitis but was also more sensitive than the culture methods. Molecular identification is the gold standard technique for the identification of corneal pathogens.

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الخلاصة

بغية تقييم كفاءة تقنية تفاعل سلسلة انزيم البلمرة (PCR) في تشخيص الاحياء المجهرية المسببة لالتهابات قرنية العين، تم جمع ٢٠ عينة من المرضى المراجعين لوحدة العيون في مستشفى ساوث هامبتن بالمملكة المتحدة وزرعها على وسط اكار الدم و اكار الجوكليت و اكار السابروود للفطريات. اجريت تقنية الـ (PCR) باستخدام بادئات عامة للانواع الفطرية والبكتيرية فضلا عن استخدام بادئات خاصة للاحياء المجهرية الاكثر شيوعا. اشارت النتائج في هذا المجال الى ان ايجابية الفحص بهذه الطريقة قد بلغت ٧٥% للحالات العشرين التي شملتها الدراسة من مرضى التهاب القرنية، وبلغت الاصابة الفطرية منها ٥٥% كانت نسبة البكتيرية ٢٠%. وبالمقابل فلم تبلغ نسبة الايجابية في الفحص سوى ٢٥% لدى استخدام الطرق الزرعية في التشخيص. اما استخدام البادئات الخاصة لاكثر الانواع البكتيرية شيوعا فقد اشار الى كون نصف الحالات البكتيرية المشخصة بتقنية (PCR) تعود الى *Staphylococcus aureus* والنصف الاخر الى *Staphylococcus epidermidis* بينما اعطى استخدام البادئات الخاصة بـ *Candida albicans* نتيجة موجبة بنسبة ٧٢% من النسبة الاصلية (٥٥%)، فيما كانت النسبة المتبقية (٢٨%) تعود الى المسببات الفطرية الاخرى. واعتمادا على ما تقدم من نتائج يمكن الاستنتاج بان تقنية الـ (PCR) كانت الطريقة الاكفأ والاسرع في تشخيص الاحياء المجهرية من بكتريا وفطريات، و التقنية الاكثر حساسية ودقة من الطرق الزرعية.

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