Int.J.Curr.Microbiol.App.Sci (2016) *5*(2): 854-860

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 5 Number 2(2016) pp. 854-860**
Journal homepage: http://www.ijcmas.com

Original Research Article doi: http://dx.doi.org/10.20546/ijcmas.2016.502.097

Utility of Polymerase Chain Reaction in Diagnosis of *Acanthamoeba* and Microsporidial Keratitis

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Keywords

Acanthamoeba, Microsporidia, keratitis, PCR.

Article Info

Accepted:
28 January 2016
Available Online:
10, February 2016

A B S T R A C T

Evaluation of the diagnostic utility of PCR in comparison to the conventional test. Descriptive study. All patients with suspected microbial keratitis presenting between October 2012 to June 2014 at the Ophthalmology OPD, JIPMER hospital. A total of 50 consecutive non-duplicated cases of keratitis were included in the study period of two years. All the samples were subjected to the conventional test like microscopy using Gram stain and modified trichrome stain, and PCR for *Acanthamoeba* and Microsporidia. Microscopy, PCR, age and sex distribution of patients with keratitis, risk factors, clinical presentation of patients. Mean age group of the patients in this study was 48.3 years (range 10-90) and majority of them were females (54%). The predominant symptom with which the patients presented in our study was pain (60%).Corneal trauma with vegetative matter was a major risk factor accounting for 20%. Out of the 50 samples, one (2%) of the specimens was positive for *Acanthamoeba* and two (4%) were positive for microsporidia by PCR, while, none of the specimens was positive by microscopy for *Acanthamoeba* and Microsporidia on Modified trichrome stained smears. Hence, this establishes the fact that PCR is superior to microscopy as it is a sensitive cum rapid method for the diagnosis of keratitis due to *Acanthamoeba* and Microsporidia.

Introduction

Acanthamoeba and Microsporidia are two opportunistic parasitic organisms which are now increasingly recognized as significant emerging cause of microbial keratitis. The reason for this rise in numbers could possibly be because of increasing awareness of the condition, gradual rise in the use of contact lens and improvement in the diagnostic modalities. Early and accurate diagnosis is the most vital step in managing *Acanthamoeba* and Microsporidial keratitis as prognosis is directly related to timely diagnosis. Current methods of diagnosing these parasites depends mainly upon their morphologic demonstration in the clinical specimen by microscopy. Culture which is the gold standard in diagnosis of *Acanthamoeba* keratitis is time consuming and requires advanced laboratory set up because of the special growth requirements

of these parasites, and thus is mainly used for epidemiological and research purposes. Electron microscopy is the gold standard for the diagnosis of microsporidial keratitis but it does not allow identification of human microsporidial pathogens to the species level(1).

Thus there is scope for diagnostic methods which are rapid, have high precision, specificity and sensitivity. PCR is a rapid and sensitive method for diagnosis and species identification of Microsporidia and *Acanthamoeba*. Results can be obtained within 72 h using PCR.High clinical utility of molecular methods like PCR in the diagnosis of keratitis has already been demonstrated by several studies(2–5). Detection of these organisms by PCR would also help us to bring out the actual incidence of these infectious organisms in our clinical setup. The following study was carried out with the objectives of detecting *Acanthamoeba*and microsporidia from corneal scrapings by PCR and compare the performance of PCR with that of microscopic examination of the corneal scrapings by modified trichrome staining so as to evaluate the diagnostic utility of PCR in a clinical set up.

Materials and Methods

This was a descriptive study conducted in the department of Microbiology, JIPMER after being approved by the Institute Research and Ethics Committee. All patients with suspected microbial keratitis attending Ophthalmology OPD were included in the study after obtaining informed consent from them.

A total of 50 consecutive non-duplicated cases of keratitis were included in the study period of two years (October 2012 to June 2014).Corneal scrapings were taken by an ophthalmologist using a sterile blade, under a slit lamp biomicroscope. Smears of corneal scrapings were collected for microscopy for preparation of KOH mount, Gram stain and modified trichrome staining. Scrapings were also collected in 0.2 ml of sterile phosphate buffer saline (pH 7.2) and stored at -20◦C for PCR.

Modified Trichrome Staining of the Smear

The corneal smear slides were stained by the Kokoskin's modification of the modified trichrome staining method (Weber green)(6).Smear was first fixed with absolute methanol for 5 minutes, the slide was then covered with modified trichrome stain for 10 min at 50◦C, rinsed with acid alcohol for 10 seconds and dipped in 95% alcohol several times and later placed in 95% alcohol for 5 minutes. This was followed by dipping the slide in 100% alcohol for 10 minutes. Finally the slide was placed in xylene substitute for 10 minutes. The air dried smear was examined under oil immersion.

Extraction of DNA from the Corneal Scrapings

DNA was extracted from the corneal scrapings collected in phosphate buffered saline (pH 7.2) using the QIAamp DNA Mini Kit (QIAGEN) as per the manufacturer's instructions. All the extracted DNA were stored at -20◦C.

Genus Specific PCR

The genus specific PCR for *Acanthamoeba* was performed as per the method proposed by Niyyati*et. al*,(7), and by Savitri*et. al*,(4) for Microsporidia with slight modifications (Table 1).

Briefly, conventional PCR was performed on the thermocycler (Eppendorf Mastercycler gradient thermocycler, Germany) for *Acanthamoeba* using a final reaction mixture of 30µl with 0.6µM concentration of primers and 10µl of DNA. The following cycling conditions were used: initial denaturation at 95◦C for 2 minutes, first step-denaturation at 95◦C for 30 seconds, annealing at 50◦C for 30 seconds, and extension at 72◦C for 30 seconds, for 32 cycles. A second set of conventional PCR was performed for Microsporidia using a final reaction mixture 50µl with 10µM concentration of primers and 10µl of DNA. The following cycling conditions were used: initial denaturation at 94◦ C for 10 minutes, first step denaturation at 94◦C for 1 minute, annealing at 54◦C for 2 minutes, and extension at 72◦C for 1 minute, for 35 cycles. The respective amplicons were detected by electrophoresis on agarose gel (2%) with ethidium bromide and visualized with the help of the Gel documentation system (Biorad Gel Doc XR system, USA), specific bands between 400-550 bp were looked for in case of *Acanthamoeba* and between 250-280 bp for Microsporidia.

Results and Discussion

The highest number of patients 12/50 (24%) were found to be above 60 years of age (Figure 1). Mean age group of the patients was 48.3 years (range 10-90). Majority of the cases in the study were females (54%).

Corneal trauma with vegetative matter (20%) was the most important risk factor which was observed in this study. None had ever used contact lens. The most common symptom with which the patients presented in our study was pain (60%) (Table 2).

The results of the various microscopic tests performed on the corneal scrapings are tabulated below (Table 3).

Of 50, one (2%) was positive for *Acanthamoeba* and 2 (4%) were positive for Microsporidia by PCR. The samples were also inoculated simultaneously in blood agar and sabarouds dextrose agar to rule out any bacterial and fungal pathogen causing keratitis.

Acanthamoeba and Microsporidia are rare etiological agents of keratitis and can cause outbreaks (8–10). The incidence of these agents may be falsely low because of lack of suspicion and less sensitive diagnostic methods. Most of the laboratories rely on microscopic methods for detection of Microsporidia and *Acanthamoeba* from clinical samples in cases of keratitis as culture is tedious, time consuming and expensive. Microscopic staining techniques for the detection of microsporidia and *Acanthamoeba*are subjective, require expertise and cannot determine the species of the infecting organism. In addition to the above drawbacks, the species of these organisms cannot be determined by the staining techniques. Newer methods like PCR are therefore replacing the microscopic tests because of their improved sensitivity, rapid nature, and ability to differentiate the species and to detect even non-viable organisms.

The clinical manifestations of *Acanthamoeba* and microsporidial keratitis are delayed, vague and protean, masquerading as those caused by other infectious agents. Most of the patients (90%) in this study presented within a week of the clinical manifestations. The most common clinical presentation was ocular pain (60%), (table 2).The involvement of the cornea was superficial in majority of the patients.

In 48% of the cases, the cause of keratitis could not be established despite our sincere efforts. Though, we had selected cases based on their clinical presentation and had

excluded suspected viral keratitis cases, some of the cases studied may have had an underlying undetected viral infection. All specimens were negative by modified trichrome staining for cysts of *Acanthamoeba* and spores of Microsporidia. This could be due to low load of the organisms in the scrapings collected.

Previous studies performed in south India have found a prevalence of 0.9% for *Acanthamoeba* (8) and (0.4-0.5%) for Microsporidia (4,18). One (2%) of the specimens was positive for *Acanthamoeba* and two (4%) were positive for Microsporidia by PCR in this study. The low prevalence of these organisms necessitates the use of a larger population in order to find out the prevalence, positive and negative predictive values.

Table.1 Genus Specific Pcr Primers For Acanthamoeba (7) and Pan-Microsporidial Primers Used For Microsporidia (4)

Organism	Primer sequence (5'-3')	Product size (bp)
Acanthamoebaspp	F- GGCCCAGATCGTTTACCGTGAA	423-551
	R-TCTCACAAGCTGCTAGGGGAGTCA	
Microsporidia	F-CACCAGGTTGATTCTGCCTGAC	250-280
	R--CCTCTCCGGAACCAAACCCTG	*E.cuniculi*:268 bp
		V.corneae: 250 bp
		E.hellem: 279 bp
		E.intestinalis:270bp

Table.2 Clinical Manifestations Observed in the Study

Signs / symptoms	Number of patients (%)
Pain	30 (60)
Decrease in vision	25 (50)
Photophobia	13 (26)
Foreign body sensation	6(12)
Redness	24 (48)
Watering	11 (22)
Mucopurulent discharge	10 (20)
Congestion	8(16)
Edema	3(6)

Table.3 Results of Various Microscopic Tests Done on the Corneal Scrapings

Test	Number of specimens Positive (%)	Number of specimen Negative (%)
Grams for bacteria	11 (22)	39 (78)
KOH for fungal elements	5 (10)	45 (90)
Modified trichrome stain for *Acanthamoeba* and Microsporidia	0	50

Table.4 Results Obtained by Culture for Bacteria/Fungi

Tests	Number of specimens Positive (%)	Number of specimen Negative (%)
Bacterial culture	**15 (30)**	**35 (70)**
Pseudomonas aeruginosa	12(24)	
Non-fermenting Gram negative bacilli	1(2)	
Streptococcus pneumoniae	1(2)	
Staphylococcus aureus	1(2)	
Fungal culture	**8 (16)**	**42 (84)**
Aspergillus spp	2(4)	
Fusarium spp	2(4)	
Aureobasidium pullulans	2(4)	
Cladosporiumspp	1(2)	
Penicilliumspp	1(2)	

Genus level identification of *Acanthamoeba* species can be accomplished by PCR based on distinctive features of trophozoite and cyst(11). Usefulness of PCR as an sensitive and specific diagnostic tool for the demonstration of *Acanthamoeba* has been proved by several studies. Vodkin *et al.*, (12) were the first to use PCR for the genus-specific detection of *Acanthamoeba* targeting the 18S rDNA using the primer pair ACARNA f1383 and ACARNA r1655.

The study of Shroeder *et al.*,(13) demonstrated the usefulness of 18S rDNA PCR amplimers ASA.S1 and GTSA.B1 obtained with primers JDP1, JDP2 and CRN5 and 1137 respectively. JDP1 and JDP2 are known for their high specificity for the genus Acanthamoeba while GTSA.B1 is useful for identification of individual genotypes. Lehmann *et al.*, (3) used genus-specific primers P1 and P2 which amplifies a 253-bp amplicon, to demonstrate the sensitivity of the clinical PCR. Small-subunit rRNA sequences are used for species-specific detection of organisms (14). Apart from small-subunit rRNA, large subunit rRNA and the intergenic spacer region can also be used as a target(4). Panmicrosporidial primers (V1 and PMP2) and species specific primers (V1-EB450 and V1-SI500) can be used for detecting microsporidia in clinical specimen (15). Using panmicrosporidial primers sensitivity and specificity was demonstrated to be 83% of 98% respectively(4). Utility of PCR for diagnosis of microsporidial keratitis giving a higher sensitivity than traditional cytologic and histologic detection methods have already been studied(16).Thus increased usage of molecular techniques for the diagnosis of ocular infections with *Acanthamoeba* and Microsporidia will be a better option for diagnosis and management of keratitis cases, due to the high accuracy of these tests. In our study we performed smear examination which was followed by genus specific PCR for detection of *Acanthamoeba* and PCR using pan-microsporidian primers for Microsporidia on all the samples. Out of the total 50 cases, majority (54%) were females.

Corneal trauma with vegetative matter was a major risk factor accounting for 20% of the cases. Contact lens usage was not observed

in this study as majority of the patients were from rural areas. Many studies conducted in India have already shown that contact lens usage is not an important risk factor in our country(17–19).

Though commonly known to cause self-limiting or latent disease, they do have the potential to cause devastating ocular condition. It is therefore important to establish proper clinical judgment and to have high level of suspicion for these organisms in cases with negative cultures for bacteria or fungi as these organisms require treatment with totally different class of drugs which do not form a part of the routine empirical therapy advocated for the cases of microbial keratitis.

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How to cite this article:

Namrata Krishna Bhosale, Subhash Chandra Parija, Jharna Mandal and Shashi Ahuja. 2016. Utility of Polymerase Chain Reaction in Diagnosis of Acanthamoeba and Microsporidial Keratitis. *Int.J.Curr.Microbiol.App.Sci.* 5(2): 854-860.
doi: http://dx.doi.org/10.20546/ijcmas.2016.502.097