

# Evaluation of PCR Assay over Culture for the Detection of *Acanthamoeba Spp.* In Amoebic Keratitis and Amoebic Meningitis/ Meningoencephalitis Patients in India

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## Research Article

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## Abstract

**Aims:** Evaluation of PCR assay over culture for the detection of *Acanthamoeba* spp. in corneal scrapings of suspected amoebic keratitis patients and CSF from suspected amoebic meningitis/ meningoencephalitis patients.

**Methods and Results:** Corneal scrapings and CSF were collected from 183 and 149 patients suspected of having amoebic Keratitis and *Acanthamoeba* meningitis/ meningoencephalitis (AME) respectively from 2010 to 2015. A part of the specimen (corneal scraping and CSF) was inoculated onto a 2% non-nutrient agar plate overlaid with *E. coli*. for culture and a second part of the specimen was placed in 500µl PBS buffer (pH 7.4) for molecular diagnosis. *Acanthamoeba* spp. could be detected in culture from 29(15.84%) cases, by PCR assay from 32(17.48%) cases in suspected amoebic keratitis patients. Likewise out of 149 suspected patients of *Acanthamoeba* meningitis/ meningoencephalitis 10(06.71%) cases were positive in culture where as 11(07.38%) were positives by PCR assay.

**Conclusion:** PCR assay was found to be more sensitive and specific than culture for detection of *Acanthamoeba* spp. in corneal scrapings of suspected amoebic Keratitis patients and CSF collected from suspected AME patients.

**Significance and Impact of Study:** PCR assay should be performed along with culture (gold standard) for efficient laboratory diagnosis of *Acanthamoeba* spp. in patient samples.

**Keywords:** Acanthamoeba keratitis; Acanthamoeba meningitis/ meningoencephalitis; 18S rDNA PCR; PCR assay of Acanthamoeba spp

**Abbreviations:** AK: *Acanthamoeba* keratitis; SLE: Systemic lupus erythematosus; ASA.S1: Acanthamoeba specific amplimer.

## Introduction

*Acanthamoeba* keratitis (AK) is an acute; sight threatening infection of cornea, if untreated can lead to permanent blindness [1]. Previously *Acanthamoeba* keratitis was considered to be a rare entity but in recent years due to development of advanced diagnostic procedures the magnitude of *Acanthamoeba* keratitis has increased rapidly across the world [2-5]. In developed countries most of the reported cases were seen to be associated with the use of soft contact lens where as in developing countries like India most reported cases were seen to be associated with non-contact lens wearers rather than the contact lens wearers [4-6].

*Acanthamoeba* meningitis/ meningoencephalitis is a rare and insidious, chronic infection of the central nervous system in persons with compromised metabolic, physiologic, or immunologic functions due to either AIDS or diabetes or Systemic lupus erythematosus (SLE) or undergone organ transplantation or in patients receiving chemotherapy/ radiotherapy etc [7-9]. Apart from all these cases there have been a few reported cases of *Acanthamoeba* meningitis/ meningoencephalitis infections in healthy individuals with no known previous immunodeficiency [10,11].

An early diagnosis of *Acanthamoeba* keratitis and *Acanthamoeba* meningitis/ meningoencephalitis infections increases the prognosis. For laboratory diagnosis, culture on 2% non-nutrient agar plate overlaid with *E.coli.* and molecular diagnosis using PCR assay are usually used worldwide. Sometimes some positive cases of these two infections are misdiagnosed due to use of only culture (gold standard) for routine laboratory diagnosis. PCR assay for amplification of "*Acanthamoeba* specific amplimer (ASA.S1)", a 464bp region within the 18S rRNA gene was found suitable for routine molecular diagnosis [12]. A major advantage of choosing this region for PCR assay is that, it detects all known *Acanthamoeba* subgroups [12]. The present study was aimed to evaluate the usefulness of PCR assay over culture in a clinical

setting with a large number of amoebic keratitis and amoebic meningitis/ meningoencephalitis patients for the detection of *Acanthamoeba* spp.

## Materials and Methods

### Patient Selection and Collection of Clinical Specimens

Corneal scrapings were collected by the ophthalmologists from 183 patients clinically suspected of having *Acanthamoeba* Keratitis with symptoms of unilateral red eye, foreign body sensation, blurred vision, severe unbearable pain, ring shaped stromal infiltrates and photophobia reporting to the Outpatient department/ casualty services of Dr R. P. Centre for Ophthalmic Sciences, New delhi from 2010 to 2015. Likewise CSF was collected by the neurologists from 149 patients clinically suspected of having *Acanthamoeba* meningitis/ meningoencephalitis with symptoms of seizures, aphasia, ataxia, altered mental state and lethargy reporting to the Outpatient department/ casualty services of Neurosciences centre, All India Institute of Medical Sciences, New Delhi, India from 2010 to 2015. A part of the collected specimen (corneal scraping or CSF) was inoculated onto a 2% non-nutrient agar plate overlaid with *E. coli.*, for culture and a second part of the specimen (corneal scraping) was placed in 500µl PBS buffer (pH 7.4) for PCR assay. CSF was directly taken for molecular diagnosis by PCR assay.

### Isolation of *Acanthamoeba* spp. in culture

Inoculated 2% non-nutrient agar plates were sealed and incubated at 30°C for 14 days. Plates were examined regularly post incubation and observed under a light microscope (10X & 40X objectives) (Nikon, Japan) for the appearance of growth of *Acanthamoeba* spp. over agar surface.

### PCR Assay

DNA was isolated from corneal scrapings (collected in 500µl PBS buffer) and CSF using QIAmp DNA Mini Kit (QIAGEN), following the manufacturer's instructions, and used for PCR assays. PCR assay for amplification of the genus specific *Acanthamoeba* specific amplimer (ASA.S1) region of 18S rRNA gene (~464bp) was performed with

the forward primer JDP1 (5'-GGCCAGATCGTTTACCGTGAA-3') and reverse primer JDP2 (5'-TCTCACAAGCTGCTAGGGGAGTCA-3')[12]. PCR reaction mix of 20µl was prepared with 1X reaction buffer(Fermentas), 2.5mM MgCl<sub>2</sub>, 200µM dNTPs (Fermentas), 0.4 µM forward and reverse primers (IDT), 1.25U *Taq* Polymerase (Fermentas) and milliQ water q.s. 5µl of extracted DNA (~0.5ng)was added to the reaction mixture and processed for PCR assay. PCR assay was performed in a thermal cycler (Applied Bio system, USA) with the temperature profile of initial denaturation at 94°C for 5mins, followed by denaturation at 94°C for 1min, primer annealing at 61°C for 1min, strand elongation at 72°C for 1min for 35 cycles, with the final elongation at 72°C for 10mins. Reaction mixture with 5µl distilled water was used as a negative control and reaction mixture with DNA isolated from known isolates of *Acanthamoeba* spp. was used as a positive control in PCR assay. Amplified PCR products were electrophoresed on 1.5% agarose gel and was visualised under a Gel documentation system (Syngene).

### Statistical Analysis

Diagnostic data (culture and PCR assay) obtained from 183 patients clinically suspected of having *Acanthamoeba* keratitis and 149 patients clinically suspected of having *Acanthamoeba* meningitis/ meningoencephalitis were used for determining sensitivity, specificity, positive predictive values (PPV), and negative predictive values (NPV) of PCR assay using culture results as the "gold standard". Formulas used for calculation are as follows: sensitivity = (number of true positives/total number of true positives + total number of false negative) X 100, specificity = (number of true negatives//total number of true negative + total number of false positives) X 100, PPV = (total number of true positives/ total number of true positives + total number of false positives) X 100, NPV = (Total number of true negatives/total number of true negatives + total number of false negatives) X 100.

### Results

Age & sex profile of 183 patients clinically suspected of having *Acanthamoeba* keratitis and 149 patients clinically suspected of having *Acanthamoeba* meningitis/ meningoencephalitis included in this study are given in

(Tables 1 & 2) respectively. Of the 183 suspected *Acanthamoeba* keratitis patients 109 were male and 74 were female. Likewise of the 149 suspected *Acanthamoeba* meningitis/ meningoencephalitis patients 100 were male and 49 were female.

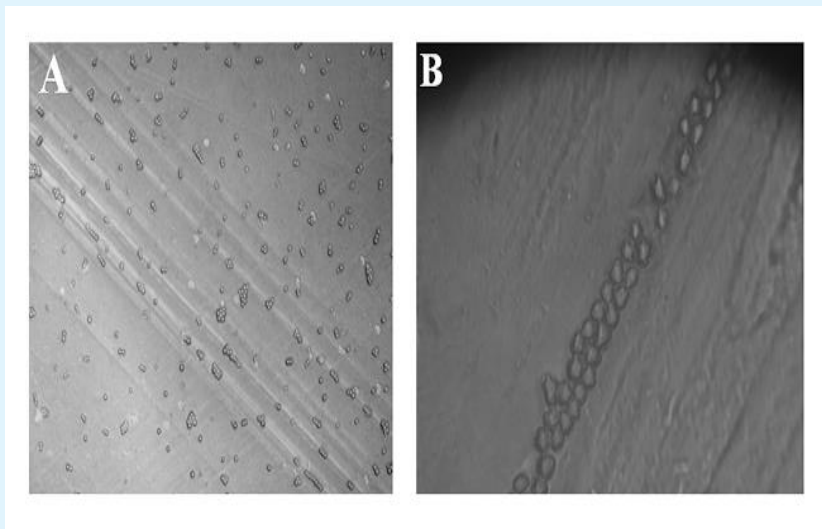
Age Group of Patients (Yrs)	No. of Samples
0-10	6
11-20	26
21-30	43
31-40	33
41-50	30
51-60	26
61-70	13
71-80	05
81-90	01

**Table 1:** Age distribution of 183 patients with suspected *Acanthamoeba* keratitis.

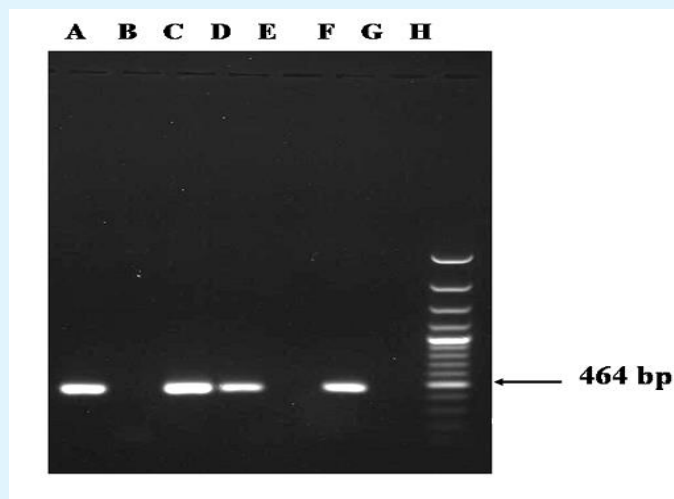
Age Group of Patients (Yrs)	No. of Samples
0-10	49
20-Nov	40
21-30	19
31-40	14
41-50	10
51-60	8
61-70	5
71-80	3
81-90	1

**Table 2:** Age distribution of 149 patients with suspected *Acanthamoeba* meningitis/ meningoencephalitis.

*Acanthamoeba* spp. were visible in the culture on 2% non nutrient agar plate overlaid with *E.coli*. using an inverted microscope (Nikon) after 4 days and covered the entire agar surface after 7 days (Figure 1A). Nucleotide sequences for ASA.S1 region of 18s rRNA gene produced clear band of ~460 bp length when electrophoresed in 1.5% agarose gel for all positive specimens (Figure 1B).



**Figure 1:** Positive culture of the *Acanthamoeba* spp. as visualized by inverted microscopy (A) with 10X objective (B) with 40X objective.



**Figure 2:** PCR amplified product of ~460 bp length of ASA.S1 region of 18S rRNA gene of *Acanthamoeba* spp. obtained from corneal scrapings of suspected amoebic keratitis patients.

{A=Positive Control ; B=Negative Control (Distilled water); C=Positive isolate; D=Positive isolate; E=Negative isolate; F=Positive isolate; G=Negative isolate; H=DNA ladder (100bps)}

Of the 183 patients clinically suspected of having *Acanthamoeba* keratitis, 29(15.84%) were found to be culture positive for *Acanthamoeba* spp., 32(17.48%) were positive in PCR assay, and 28(15.30%) were positive by both culture & PCR assays ( $P < 0.0001$ , P-give 95%

confidence interval) (Table 3). Likewise out of 149 patients clinically suspected of having *Acanthamoeba* meningitis/ meningoencephalitis 10(06.71%) were found positive by culture, 11(07.38%) were positive by PCR assay and 10(06.71%) were positive by both culture and

PCR assay ( $P < 0.0001$ , P-give 95% confidence interval) (Table 4). P-value was calculated with two tailed Fisher's exact test.

Culture (Gold stand.)	PCR Assay	
	(Positive)	(Negative)
Positive	28	1
	-15.30%	-0.54%
Negative	4	150
	-2.10%	-81.96%

**Table 3:** 2X2 table of culture and PCR results for 183 suspected patients of having amoebic keratitis.

Culture (Gold stand.)	PCR Assay	
	(Positive)	(Negative)
Positive	10	0
	-6.71%	0.00%
Negative	1	138
	-0.67%	-92.61%

**Table 4:** 2X2 table of culture and PCR results for 149 suspected patients of having *Acanthamoeba* meningitis/ meningoencephalitis.

Sensitivity and specificity of PCR assay in case of suspected amoebic keratitis patients were found to be 96.55% and 97.40% respectively with culture as the gold standard. Similarly sensitivity and specificity of PCR assay in case *Acanthamoeba* meningitis/ meningoencephalitis patients were found to be 100.00% and 99.28% respectively with culture as the gold standard. Positive predictive value (PPV) and Negative predictive value (NPV) of PCR assay in case of amoebic keratitis was found to be 87.50% and 100% respectively and in case of *Acanthamoeba* meningitis/ meningoencephalitis it was found to be 90.90% and 100% respectively.

## Discussion

*Acanthamoeba* spp. are mostly detected from clinical specimens by three laboratory diagnostic methods such as direct examination by several staining procedures, culture and PCR assay [13,14]. Among all these three methods, staining and PCR assay can provide a rapid detection of *Acanthamoeba* spp. as compared to culture [15,13]. In a pilot study it was reported that, *Acanthamoeba* spp. was misdiagnosed in 60-70% of direct examination by staining, hence this method cannot be only implemented for accurate laboratory diagnosis [13]. However culture on 2% non-nutrient agar plate overlaid

with *E.coli* is the easiest and most widely used method for laboratory diagnosis of *Acanthamoeba* spp. which is also considered as the gold standard worldwide [16]. This is probably due to its low cost, easy to handle and can be carried out by a person without much more technical knowledge in molecular biology. There is still a drawback with culture which takes a longer incubation time (7-14 days) for reporting the results and there may some false positive cases in case of contact lens due to contamination as reported from some previous studies [16,13]. With the introduction of PCR assay into laboratory diagnosis it was widely accepted along with culture and staining. The drawbacks of culture and staining were overcome by PCR assay in terms of sensitivity, specificity, rapidity and accuracy.

Several regions of nuclear DNA and ribosomal DNA were chosen for molecular diagnosis by PCR assay, among which 18S rRNA gene-based PCR assay is widely used for its high sensitivity and specificity. Vodkin *et al.* were the first to use PCR assay for the detection of several strains *Acanthamoeba* spp. in clinical specimens, using primer pairs ACARNA for1383 and ACARNA.rev1655 that amplifies a 272bp region of 18s rDNA [17]. These pair of primers were successful in distinguishing *Acanthamoeba* from *Naegleria*. Later Lehmann, *et al.* also tested with another set of primers P1GP for 2379 and P1GP.Rev2632, to amplify a 253 bp region of 18S rDNA gene of *Acanthamoeba* spp. [16]. Analyzing complete 18S rDNA gene sequences Schroeder, *et al.* [12] had shown that, the above two primer pairs could also amplify 18S rDNA gene sequences of related amoebas i.e., *Balamuthia* and *Hartmanella* spp. Hence he designed a new set of genus specific primers i.e. JDP1 and JDP2 which could amplify a ~464 bp region of 18S rDNA of *Acanthamoeba* spp. [12]. These pair of primers were genus specific for *Acanthamoeba* and could not amplify from DNA of closely related amoebae. From that time onwards these set of primers are widely used in laboratory diagnosis of all species of *Acanthamoeba* all over the world [18,19,4].

In a study it was reported that, *Acanthamoeba* spp. could be detected by PCR assay in 71.4% of suspected amoebic keratitis patients whereas culture was positive in only 14.3% of the isolates [20]. In another pilot study to compare culture vs. PCR it was reported that, out of 80 patients of suspected amoebic keratitis, 4 additional positive cases were obtained by PCR assay than culture [21]. The present study also corroborates with the previous studies, where we found 3 more *Acanthamoeba* positive cases in amoebic keratitis and 1 more positive



case in *Acanthamoeba* meningitis/ meningoencephalitis by PCR assay than culture on 2% non-nutrient agar plate.

Lehmann, *et al.* had reported the advantage of PCR assay over culture in terms of sensitivity and specificity [16]. They reported a sensitivity of 84% and 66% for the PCR assay in diagnosis of strains of *Acanthamoeba* spp. from corneal scrapings and tear specimens respectively in comparison to just 53% by the traditional culture method [16]. In another study it was reported that, PCR assay has a higher sensitivity of 87.5% for detection of *Acanthamoeba* spp. from corneal scrapings as against culture which was 70% [4]. In a pilot study conducted by Niyiyati, et al. [20]. It was reported that, sensitivity and specificity of PCR-based method was 71.4% and 100% respectively taking culture as the gold standard in 28 suspected *Acanthamoeba* keratitis patients [20]. The present study corroborates with the previous studies which showed PCR assay is having a higher sensitivity and specificity in detecting *Acanthamoeba* spp. from corneal scrapings of suspected amoebic keratitis patients and CSF of suspected *Acanthamoeba* meningitis/ meningoencephalitis patients. Apart from sensitivity and specificity, PCR assay was found to having high PPV and NPV values which further confirms that "PCR assay is a good diagnostic test for detecting *Acanthamoeba* spp. in clinical specimens". Therefore at present both culture and PCR assay are being widely used for laboratory diagnosis of *Acanthamoeba* keratitis and *Acanthamoeba* meningitis/ meningoencephalitis to maximise detection [4,22,12,18].

Summarising the whole study, we evaluated the effectiveness of PCR assay over culture in detecting *Acanthamoeba* spp. from corneal scrapings of suspected amoebic keratitis patients and CSF from suspected *Acanthamoeba* meningitis/ meningoencephalitis patients. From our pilot study we suggest that, PCR assay should be used to supplement the conventional culture for the detection of amoebic keratitis and *Acanthamoeba* meningitis/ meningoencephalitis infections [23].

## Conclusions

PCR assay was found to be more sensitive and specific than culture for detection of *Acanthamoeba* spp. in corneal scrapings of suspected amoebic Keratitis patients and CSF collected from suspected AME patients. PCR assay should be performed along with culture (gold standard) for efficient laboratory diagnosis of *Acanthamoeba* spp. in patient samples.

## Ethical Approval

All procedures performed in this study involving human participants were in accordance with the ethical standards of the institute (AIIMS, New Delhi) and ethical clearance was obtained from the institute.

## Acknowledgments

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## Conflict of Interests

The authors declare that there are no conflicts of interest related to this work.

## Contributorship

HB: Performed all laboratory experiments and drafted the manuscript. AP: Clinical team leader did clinical examinations and provided specimens. PB: Did clinical work and provided specimens. MT: Clinical team leader did clinical examinations and provided clinical specimens. GS: Conceived the idea, arranged the funding and supervised the work, shaped the drafted manuscript.

## Informed Consent

Consent was obtained from all individual participants included in the study.

## Funding and all other required documents

There are no funding agencies to report for this submission.

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