

## Use of 18S rRNA Gene-Based PCR Assay for Diagnosis of *Acanthamoeba* Keratitis in Non-Contact Lens Wearers in India

Gunisha Pasricha,<sup>1</sup> Savitri Sharma,<sup>1</sup> Prashant Garg,<sup>2</sup> and Ramesh K. Aggarwal<sup>3\*</sup>

*Jhaveri Microbiology Centre, Prof. Brien Holden Eye Research Centre, Hyderabad Eye Research Foundation,<sup>1</sup> and Cornea Service, L. V. Prasad Eye Institute,<sup>2</sup> and Centre for Cellular and Molecular Biology,<sup>3</sup> Hyderabad, India*

Received 9 December 2002/Returned for modification 23 January 2003/Accepted 6 April 2003

**Identification of *Acanthamoeba* cysts and trophozoites in ocular tissues requires considerable expertise and is often time-consuming. An 18S rRNA gene-based PCR test, highly specific for the genus *Acanthamoeba*, has recently been reported in the molecular diagnosis of *Acanthamoeba* keratitis. This PCR assay was compared with conventional microbiological tests for the diagnosis of *Acanthamoeba* keratitis. In a pilot study, the PCR conditions with modifications were first tested on corneal scrapings from patients with culture-proven non-contact lens-related *Acanthamoeba*, bacterial, and fungal keratitis. This was followed by testing of corneal scrapings from 53 consecutive cases of microbial keratitis to determine sensitivity, specificity, and predictive values of the assay. All corneal scrapings from patients with proven *Acanthamoeba* keratitis showed a 463-bp amplicon, while no amplicon was obtained from patients with bacterial or fungal keratitis. Some of these amplified products were sequenced and compared with EMBL database reference sequences to validate these to be of *Acanthamoeba* origin. Out of 53 consecutive cases of microbial keratitis included for evaluating the PCR, 10 (18.9%) cases were diagnosed as *Acanthamoeba* keratitis on the basis of combined results of culture, smear, and PCR of corneal scrapings. Based on culture results as the “gold standard,” the sensitivity of PCR was the same as that of the smear (87.5%); however, the specificity and the positive and negative predictive values of PCR were marginally higher than the smear examination (97.8 versus 95.6%, 87.5 versus 77.8%, and 97.8 versus 97.7%) although the difference was not significant. This study confirms the efficacy of the PCR assay and is the first study to evaluate a PCR-based assay against conventional methods of diagnosis in a clinical setting.**

*Acanthamoeba* keratitis has been described primarily from developed countries of the world, with several studies suggesting soft contact lens wear as the greatest risk factor. In contrast, the reports from India and other developing countries are few and have mainly been in non-contact lens wearers (10). This low incidence of *Acanthamoeba* keratitis in developing countries may not be a true picture and calls for detailed epidemiological studies. In all probability, the reported low incidence is due to lack of sensitive diagnostic tools, low awareness, and probably the belief that the disease is related mainly to contact lens wear—a factor usually absent in most cases of keratitis from this part of the world (10). Although we have reported a number of cases, our reports were based on microscopy of the corneal scrapings and culture on nonnutrient agar with *Escherichia coli* overlay (10, 11). Between February 1991 and June 2002, we diagnosed and treated 168 cases of *Acanthamoeba* keratitis. In 25 (15%) of 168 cases, microscopy of the corneal scrapings with calcofluor white, Gram, and Giemsa staining was negative and the diagnosis was based on culture. It is well known that direct smear examination procedures provide immediate diagnosis while culture may take 1 to 10 days (average in our series, 3.5 days). Therefore, in the face of negative smears, a delay of several days in diagnosis is involved, thus leading to a delay in instituting specific therapy.

Several investigators have demonstrated the usefulness of

molecular methods for detection and identification of *Acanthamoeba* (9). These methods could be suitable for both clinical and epidemiological purposes; therefore, they need to be reliable and sensitive. PCRs with corneal scrapings, corneal epithelial biopsy specimens, and tear samples for diagnosis of *Acanthamoeba* keratitis have shown promising results (6). The technique of fluorescent in situ hybridization has also been successfully employed for the purpose (13).

Of the several primers used heretofore, Schroeder et al. (9) described a PCR assay using 18S rRNA gene (rDNA)-based primers as being most specific for the genus *Acanthamoeba*. They employed the PCR for the detection of *Acanthamoeba* DNA in corneal scrapings from a limited number of patients. This study aims to evaluate this PCR assay in a clinical setting in an ocular microbiology laboratory with a high volume of microbial keratitis patients and compare the results with those of conventional microbiological methods for the diagnosis of *Acanthamoeba* keratitis.

### MATERIALS AND METHODS

**Reference samples.** *A. castellanii* (ATCC 50370) was obtained from American Type Culture Collection, Manassas, Va., and maintained in axenic PYG (protease-peptone–yeast–glucose) culture. Cultures of bacteria (*Pseudomonas aeruginosa*), fungus (*Aspergillus* spp.), and virus (herpes simplex virus) were clinical isolates from corneal scrapings processed in our laboratory. Human leukocytes were obtained from blood donated by a volunteer.

**Patients.** All patients seen at L. V. Prasad Eye Institute with suspected microbial keratitis are routinely required to undergo microbiological investigations before institution of therapy. Patients with suspected nonviral keratitis are investigated for the presence of bacteria, fungi or *Acanthamoeba* by using a common protocol that involves collection of corneal scrapings for smears and cultures. We have described these procedures in detail in an earlier publication (5).

\* Corresponding author. Mailing address: Centre for Cellular and Molecular Biology, Uppal Rd., Tarnaka, Hyderabad-500 007, India. Phone: 91-40-27192643. Fax: 91-40-27160591. E-mail: rameshka@ccmb.res.in.

In general, smears of corneal scrapings are routinely examined after staining with (i) potassium hydroxide plus calcofluor white, (ii) Gram stain, and (iii) Giemsa stain, and the results become available within 15 to 30 min. For a pilot study, based on the smear results (later confirmed by culture), corneal scrapings were collected from 30 patients deemed to have either *Acanthamoeba*, bacterial, or fungal keratitis. The corneal scrapings were collected in 1 ml of phosphate-buffered saline, pH 7.2, and stored at  $-20^{\circ}\text{C}$  until tested by PCR.

Corneal scrapings were also collected in a similar manner from 53 consecutive cases of suspected microbial keratitis patients seen between March 2002 and June 2002 and stored at  $-20^{\circ}\text{C}$  until tested by PCR. Patients with little corneal infiltrate or those who were otherwise uncooperative were excluded from the study as additional corneal scrapings could not be collected.

**DNA extraction from reference samples and corneal scrapings.** The genomic DNA of *A. castellanii* and few clinical isolates of *Acanthamoeba* (obtained in our pilot study from suspected cases of keratitis) were isolated using the UNSET procedure (4). Briefly, the harvested cells were washed twice using 5 ml of phosphate-buffered saline and resuspended in 0.5 ml of UNSET lysis buffer for DNA isolation. The aqueous lysate was extracted twice with 0.5 ml of phenol-chloroform-isoamyl alcohol (25:24:1). The DNA was finally precipitated using 0.1 vol of 3 M NaCl and 2 vol of ethanol and resuspended in 50  $\mu\text{l}$  of double autoclaved milliQ water. The extraction of DNA from *P. aeruginosa*, *Aspergillus* species, herpes simplex virus, and human leukocytes followed the procedures described elsewhere (1, 2, 8).

DNA extraction from corneal scrapings was done by a procedure similar to the one described above except that the organic phase extraction was done only once and final DNA was dissolved in only 30  $\mu\text{l}$  of double-autoclaved milliQ water.

**PCR analysis.** The sequence of the 18S rDNA primer, used in this study, was obtained from Thomas J. Byers (The Ohio State University, Columbus) and consisted of forward primer 5'-GGCCAGATCGTTTACCGTGAA-3' and reverse primer 5'-TCTCACAAGCTGCTAGGGGAGTCA-3'. These primer sequences correspond to bp 928 to 949 and bp 1367 to 1390 bp, respectively, of *A. castellanii* ATCC 50374 18S rDNA (EMBL accession no.U07413). The primers were synthesized at the Centre for Cellular and Molecular Biology, Hyderabad, India. All PCRs were carried out in a laminar-flow hood after 30 min of UV irradiation to decontaminate surfaces and all supplies within the hood. Presterilized PCR tubes, double autoclaved milliQ water, and positive-displacement tips and pipettes were used to reduce the possibility of contamination. The primer pairs were tested initially for amplification of *A. castellanii*; clinical isolates of *Acanthamoeba*, *P. aeruginosa*, *Aspergillus* species, and herpes simplex virus; and human leukocyte DNA. The PCR conditions were modified with regard to amplification profile and  $\text{MgCl}_2$  requirement compared to those described earlier (9). The amplification profile was  $94^{\circ}\text{C}$  for 1 min,  $72^{\circ}\text{C}$  for 1 min, and  $72^{\circ}\text{C}$  for 1 min for 40 cycles followed by a final extension step of  $61^{\circ}\text{C}$  for 5 min. Each 20- $\mu\text{l}$  PCR mixture comprised 3  $\mu\text{l}$  of DNA (in the case of corneal scraping DNA extracts) or  $\sim 10$  ng of genomic DNA, 200  $\mu\text{M}$  deoxynucleoside triphosphates, a 1 pM concentration of each primer,  $1\times$  standard PCR buffer (containing 1.5 mM  $\text{MgCl}_2$ ), and 1 U of *Taq* DNA polymerase (Gene *Taq*; MBI Fermentas, Vilnius, Lithuania). These conditions differed from the ones described by Schroeder et al. (9). Amplifications, which were performed in an MJ Research PTC 150 thermocycler. The DNA extracts of corneal scrapings of culture-confirmed *Acanthamoeba* keratitis patients were tested initially in a pilot study. On obtaining satisfactory results, corneal scrapings from consecutive patients with microbial keratitis were tested.

The PCR products were visualized by gel electrophoresis using 1.5% agarose-TAE (Tris-acetic acid-EDTA) gel containing ethidium bromide (0.5  $\mu\text{g}/\text{ml}$ ), and the results were recorded on a UV gel documentation system (UVitec Ltd., Cambridge, United Kingdom).

**DNA sequencing and comparison.** Approximately 450 bp of 18S rDNA-specific PCR products obtained from 12 clinical isolates of *Acanthamoeba* obtained in the pilot study from patients with keratitis were sequenced for both strands using a 2 pM concentration of each of the original primers used for amplification and the Big Dye Terminator sequencing kit (Applied Biosystems, Foster City, Calif.) as per the manufacturer's details. The sequencing amplification conditions were as follows:  $96^{\circ}\text{C}$  for 10 s,  $50^{\circ}\text{C}$  for 5 s, and  $60^{\circ}\text{C}$  for 4 min for 30 cycles. After PCR, the products were precipitated using 1  $\mu\text{l}$  of 3 M sodium acetate (pH 4.6) and 50  $\mu\text{l}$  of ethanol and were incubated on ice for 10 min. The pellet was recovered by centrifugation ( $18,000 \times g$  for 20 min at  $4^{\circ}\text{C}$ ), washed with 70% ethanol, dried, and dissolved in 10  $\mu\text{l}$  of diluted Hi-Di formamide (Perkin-Elmer, Applied Biosystems, Foster City, Calif.). Sequencing was performed in an ABI PRISM 3700 DNA analyzer. Raw sequences were edited and assembled using the Auto Assembler program. The sequences obtained in the study were used to identify related reference sequences using a BLASTn search, and these sequences were then retrieved from the EMBL database. All the sequences were

finally aligned and used to infer the genetic similarities using CLUSTAL-X (<http://www-igbmc.u-strasbg.fr/BioInfo/clustal>) software.

**Statistical analysis.** Diagnostic data from corneal scrapings of 53 consecutive patients with suspected microbial keratitis were used for determination of sensitivity, specificity, positive predictive values (PPV), and negative predictive values (NPV) of smear examination and PCR results using culture results as the "gold standard." The following formulae were used for calculations: sensitivity = (number of true positives/total number of culture positives)  $\times 100$ , specificity = (number of true negatives/total number of culture negatives)  $\times 100$ , PPV = (number of true positives/number of true positives + number of false positives)  $\times 100$ , NPV = (number of true negatives/number of true negatives + number of false negatives)  $\times 100$ , FP = (number of false positives/total number of culture negatives)  $\times 100$ , and FN = (number of false negatives/total number of culture positives)  $\times 100$ , where the total number of culture positives is the number of true positives plus the number of false negatives, the total number of culture negatives is the number of true negatives plus the number of false positives, FP is the rate of false positives, and FN is the rate of false negatives.

## RESULTS

**Specificity of 18S rDNA-based PCR.** The primers produced *Acanthamoeba*-specific amplicon (463 bp) from *A. castellanii* DNA (ATCC 50370) and clinical isolates of *Acanthamoeba* but not from bacterial, fungal, viral, and human leukocyte DNA.

Double-strand sequencing of the above 463-bp PCR-amplified products confirmed these to be *Acanthamoeba*-specific amplicons. The sequences obtained in the study (EMBL accession no. AF534143 to AF534154) were found to be most similar (95.6 to 100%) to the reference keratitis-associated pathogenic isolates or species of *Acanthamoeba*. In general these showed an average genetic dissimilarity of  $0.023 \pm 0.015$  for the amplified 18S rDNA from those of the reference sequences (AY148954, U07401, AY026249, AF019062, and U07417). Two of the sequences (AF534149 and AF534151) obtained in the study showed 100% similarity to *A. polyphaga* (AF019062) and *Acanthamoeba* sp. isolate U/E7 (AY026249).

**Pilot study on corneal scrapings.** Corneal scrapings were taken from 30 patients selected on the basis of routine smear and culture results. Twenty-one of 30 (70%) patients were diagnosed to have *Acanthamoeba* keratitis based on detection of cysts in smears and/or growth of *Acanthamoeba* in culture. Three of the 30 (10%) patients had bacterial keratitis, and 6 of the 30 (20%) had fungal keratitis. While the corneal scrapings from all 21 patients with *Acanthamoeba* keratitis showed a 463-bp amplicon in PCR, the scrapings from patients with bacterial and fungal keratitis showed no amplification. A brief summary of the clinical findings, diagnosis, and treatment outcome of 21 patients with *Acanthamoeba* keratitis included in the pilot study is given in Table 1.

**Sensitivity, specificity, and predictive values of the 18S rDNA-based PCR assay.** Fifty-three consecutive patients with suspected microbial keratitis were included for analysis of the above parameters. These patients were seen between March and June 2002, and after collection of an average of seven multiple corneal scrapings for microbiological investigation, an extra scraping was collected for PCR. Therefore, some patients with small infiltrates could not be included in the study as it was not possible to collect an extra scraping. Table 2 summarizes the demographic details, predisposing factors, and diagnosis of these patients, and Table 3 outlines the results of microbiological investigations and their correlation with PCR results on the corneal scrapings of these patients. Considering combined results of culture, smears, and PCR, 10 cases of

TABLE 1. Clinical findings, diagnosis, and treatment outcome of 21 patients with *Acanthamoeba* keratitis included in preliminary evaluation of the PCR

Characteristic	No. (%) of patients (n = 21)
<b>Demographics</b>	
Males .....	12 (57.1)
Females .....	9 (42.9)
Mean age ± SD (yr) .....	34.3 ± 14.5
Age range (yr) .....	7–63
<b>Predisposing factors</b>	
Contact lens wear .....	0
Trauma .....	6 (28.5)
Foreign body .....	5 (23.8)
Unknown .....	10 (47.6)
<b>Detection of <i>Acanthamoeba</i> cysts in corneal scraping smears</b>	
Calcofluor white .....	20 (95.2) <sup>b</sup>
Gram stain .....	18 (85.7)
Giemsa stain .....	14 (66.6)
Culture on nonnutrient agar .....	20 (95.2) <sup>c</sup>
<b>Treatment outcomes</b>	
Healed with medical therapy .....	11 (52.4)
Surgical intervention .....	3 (14.3) <sup>a</sup>
Lost to follow-up .....	7 (33.3)

<sup>a</sup> Interventions: penetrating keratoplasty (n = 1) and evisceration (n = 2).

<sup>b</sup> One sample was smear positive and culture negative.

<sup>c</sup> One sample was culture positive and smear negative.

*Acanthamoeba* keratitis out of 53 consecutive patients were identified. Culture was positive in eight of these cases. While six out of eight were positive by both smear and PCR, one was smear positive and PCR negative and one was smear negative and PCR positive (Table 4). The two culture-negative cases were positive in smears while PCR was positive in only one of them.

**DISCUSSION**

In last 11 years, we have diagnosed and treated 168 patients with *Acanthamoeba* keratitis; among whom only one was contact lens wearer. A total of 197 specimens including corneal scrapings and corneal buttons from these patients had been subjected to smear and culture examination (data not shown). Both smear and cultures were positive in 130 (66%) specimens; the smear was positive and the culture was negative in 27 (14%); the smear was negative and the culture was positive in 33 (17%); and both smear and culture were negative in eight (4%) specimens. In cases where the smear was negative and culture was positive (17%), the diagnosis was delayed for a mean of 3.5 days. In cases where both smear and culture were negative (4%), the initial diagnosis was made by having a high clinical suspicion and the final diagnosis was by detecting *Acanthamoeba* cysts by cultures or smear in repeated corneal scrapings or corneal button obtained during penetrating keratoplasty from the same patient.

In order to enhance our diagnostic capability, we decided to use a molecular diagnostic assay that may have an advantage over smear and culture and may be more sensitive and specific, as well as rapid, for the detection of *Acanthamoeba* from clinical samples. Although PCR-based assays for the detection of *Acanthamoeba* in corneal scrapings have been described, none have been tested on patients with non-contact lens-related

keratitis. We decided to evaluate the PCR assay (9), which was described to be highly specific and was based on 18S rRNA gene of *Acanthamoeba*. We evaluated this assay against the appropriate positive and negative controls and found it to be specific for *Acanthamoeba* with modifications in the assay conditions. Using the same primers, we also confirmed that the culture isolates from our patients with no history of contact lens wear were indeed *Acanthamoeba*. The specificity of the assay was further confirmed in our pilot study with corneal scrapings from all 21 cases of culture- and/or smear-positive cases of *Acanthamoeba* keratitis yielding positive result in PCR. Convinced of the results of specificity of the assay, we evaluated this test on 53 patients with suspected microbial keratitis. To the best of our knowledge this is the first study to evaluate the sensitivity, specificity, and predictive values of a PCR technique vis-à-vis a smear and culture that form the routine diagnostic tests in most ocular microbiology laboratories for the diagnosis of *Acanthamoeba* keratitis.

Our results show that the sensitivity of PCR was similar (87.5%) to that of smear results, which included three methods of smear examination such as calcofluor white, Gram stain, and Giemsa stain (Tables 3 and 4). The long experience of the microbiologists involved in examination of corneal scraping smears in this laboratory may account for the high positivity of smears. Patient samples 20 and 46 (Table 3) were culture negative but smear positive, while sample number 20 was found to be culture negative but PCR positive. It is possible that the testing of last scraping by PCR led to negative results (in 2 samples out of 10) and the sensitivity would have been higher if it was tested on initial corneal scrapings of the patients rather than the last.

On the other hand, this study found higher specificity and predictive values of the PCR assay compared to smear meth-

TABLE 2. Demographic details, predisposing factors, and microbiological diagnosis in 53 patients with microbial keratitis

Characteristic	No. (%) of patients (n = 53)
<b>Demographics</b>	
Males .....	38 (71.7)
Females .....	15 (28.3)
Mean age ± SD (yr) .....	37.92 ± 19.24
Age range (yr) .....	2–86
<b>Predisposing factors</b>	
Contact lens wear .....	0
Trauma .....	29 (54.7)
Diabetes .....	1 (01.9)
Leprosy .....	1 (01.9)
Prior surgery .....	6 (11.3)
Blepharitis .....	1 (01.9)
Lagophthalmos .....	2 (03.8)
Spheroidal degeneration .....	1 (01.9)
Unknown .....	12 (22.6)
<b>Microbiological diagnosis<sup>a</sup></b>	
Bacterial .....	17 (32.0)
Fungal .....	14 (26.4)
<i>Acanthamoeba</i> .....	7 (13.2)
Viral (HSV) .....	2 (03.8)
Bacterial + <i>Acanthamoeba</i> .....	1 (01.9)
Fungal + viral .....	1 (01.9)
Sterile (unknown) .....	11 (20.7)

<sup>a</sup> Based on culture of bacteria, fungi, or *Acanthamoeba* and antigen and/or DNA detection of herpes simplex virus (HSV) in corneal scrapings.

TABLE 3. Correlation of microbiological findings and PCR results of corneal scrapings from 53 consecutive patients<sup>a</sup>

Serial no.	Laboratory no.	Direct smear examination result(s)			Culture result(s)	PCR result
		CFW	Gram	Giemsa		
1	400/02	-	-	-	-	-
2	417/02	-	GPC	Cocci	<i>Streptococcus pneumoniae</i>	-
3	424/02	-	-	-	<i>Pseudomonas aeruginosa</i>	-
4	429/02	FF	FF	FF	<i>Aspergillus fumigatus</i>	-
5	432/02	-	GPC	Cocci	<i>Streptococcus pneumoniae</i>	-
6	490/02	Microsporidium	Microsporidium	Microsporidium	ND	-
7	503/02	-	-	-	<i>Staphylococcus epidermidis</i>	-
8	512/02	-	-	-	<i>Pseudomonas aeruginosa</i>	-
9	513/02	-	GPC	Cocci	<i>Streptococcus pneumoniae</i>	-
10	569/02	-	-	-	<i>Staphylococcus epidermidis</i>	-
11	570/02	-	-	-	-	-
12	577/02	-	-	Bacilli	<i>Pseudomonas aeruginosa</i>	-
13	603/02	Actinomycetes	Actinomycetes	Actinomycetes	<i>Nocardia asteroides</i>	-
14	604/02	-	-	-	-	-
15	607/02	<i>Acanthamoeba</i> cysts	<i>Acanthamoeba</i> cysts	<i>Acanthamoeba</i> cysts	<i>Acanthamoeba</i> spp.	+
16	632/02	FF	FF	FF	UIHF	-
17	652/02	FF	FF	FF	<i>Aspergillus niger</i>	-
18	653/02	FF	FF	FF	<i>Aspergillus niger</i>	-
19	654/02	FF	FF	FF	UIHF	-
20	661/02	-	<i>Acanthamoeba</i> cysts	ND	-	+
21	710/02	-	GPC	Cocci	<i>Streptococcus pneumoniae</i>	-
22	753/02	ND	-	ND	-	-
23	788/02	<i>Acanthamoeba</i> cysts	<i>Acanthamoeba</i> cysts	<i>Acanthamoeba</i> cysts	<i>Acanthamoeba</i> spp.	+
24	862/02	-	-	-	-	-
25	865/02	-	-	-	$\alpha$ -Hemolytic streptococci	-
26	877/02	-	-	-	-	-
27	883/02	Actinomycetes	-	-	<i>Nocardia asteroides</i>	-
28	886/02	FF	FF	FF	<i>Bipolaris</i> spp.	-
29	889/02	-	GPC	Cocci	<i>Streptococcus pneumoniae</i>	-
30	918/02	-	GPC	Cocci	<i>Streptococcus pneumoniae</i>	-
31	925/02	<i>Acanthamoeba</i> cysts	<i>Acanthamoeba</i> cysts	-	<i>Acanthamoeba</i> spp.	+
32	932/02	FF	FF	FF	<i>Fusarium</i> spp.	-
33	953/02	<i>Acanthamoeba</i> cysts	<i>Acanthamoeba</i> cysts	<i>Acanthamoeba</i> cysts	<i>Acanthamoeba</i> spp.	+
34	956/02	FF	FF	FF	<i>Aspergillus flavus</i>	-
35	995/02	FF	FF	FF	UIHF, <i>Streptococcus pneumoniae</i>	-
36	1001/02	-	GPC, GNB	Cocci, bacilli	<i>Pasteurella</i> spp.	-
37	1004/02	-	-	-	-	-
38	1009/02	FF	FF	FF	<i>Fusarium</i> spp.	-
39	1014/02	FF	FF	-	UIHF	-
40	1015/02	FF	FF	FF	<i>Aspergillus flavus</i> *	-
41	1016/02	FF	FF	FF	<i>Aspergillus flavus</i>	-
42	1046/02	FF	FF	FF	<i>Aspergillus flavus</i>	-
43	1070/02	<i>Acanthamoeba</i> cysts	<i>Acanthamoeba</i> cysts	<i>Acanthamoeba</i> cysts	<i>Acanthamoeba</i> spp.	+
44	1132/02	-	GPC	Cocci	$\alpha$ -Hemolytic streptococci	-
45	1148/02	<i>Acanthamoeba</i> cysts	<i>Acanthamoeba</i> cysts	<i>Acanthamoeba</i> cysts	<i>Acanthamoeba</i> spp.	-
46	1177/02	<i>Acanthamoeba</i> cysts	<i>Acanthamoeba</i> cysts	<i>Acanthamoeba</i> cysts	-	-
47	1201/02	-	-	-	-	-
48	1205/02	<i>Acanthamoeba</i> cysts	<i>Acanthamoeba</i> cysts	<i>Acanthamoeba</i> cysts	<i>Acanthamoeba</i> spp.	+
49	1210/22	FF	FF	FF	<i>Aspergillus fumigatus</i>	-
50	1224/02	-	GPC	Cocci	$\alpha$ -Hemolytic streptococci	-
51	1226/02	-	-	-	-	-
52	1243/02	-	-	-	<i>Acanthamoeba</i> spp. <i>Staphylococcus epidermidis</i>	+
53	1253/02	FF	FF	FF	<i>Fusarium</i> spp.	-

<sup>a</sup> Abbreviations and symbols: FF, fungal filaments; GPC, gram-positive cocci; GNB, gram-negative bacilli; UIHF, unidentified hyaline fungus; ND, not done; CFW, calcofluor white; -, negative; +, positive; \*, positive for herpes simplex virus antigen and/or DNA.

ods (Table 4), although the difference was statistically not significant. Despite repeated testing, no PCR products were seen in the corneal scrapings from patient 45 (smear and culture positive for *Acanthamoeba*) and patient 46 (smear positive for *Acanthamoeba* cysts and culture negative), which we attribute to the possible lack of *Acanthamoeba* DNA in the sample. The possibility of PCR inhibitors was ruled out in these two samples by spiking them with *Acanthamoeba* DNA and retesting them. Low DNA yield in clinical samples is

known to affect the success of PCR, especially if only mature cysts are present (9).

Apart from high sensitivity, this study demonstrates high specificity and high PPV and NPV of the PCR assay, all of which are hallmarks of a good diagnostic test. Unlike smear and culture techniques that require familiarity with the morphology of cysts and trophozoites of *Acanthamoeba*, while it does require the proper facilities, PCR does not require such expertise. Any laboratory with a molecular biology set up can

TABLE 4. Summary of smear and PCR testing of corneal scrapings for the diagnosis of *Acanthamoeba* keratitis in comparison to culture-based detection<sup>a</sup>

Diagnostic test result	Culture positive				Culture negative			
	No. of scrapings	SENS (%)	PPV (%)	FN (%)	No. of scrapings	SPEC (%)	NPV (%)	FP (%)
Smear positive	7	87.5	77.8		2			4.4
Smear negative	1			12.5	43	95.6	97.7	
PCR positive	7	87.5	87.5		1			2
PCR negative	1			12.5	44	97.8	97.8	

<sup>a</sup> Abbreviations: SENS, sensitivity; SPEC, specificity; FN, false-negative rate; FP, false-positive rate.

easily adopt the PCR assay used in this study. Moreover, smear examination techniques, especially calcofluor white, are known to miss trophozoites, and a sample with only trophozoites is likely to be labeled as smear negative (12, 13). In this study, a combination of smear and culture provided diagnoses in similar numbers of cases as smear and PCR. There are not many ocular microbiology laboratories that employ multiple smear examination protocol for corneal scrapings. Moreover, the availability of fluorescence microscopes, required for observation of smears stained with calcofluor white, is also not very common owing to its high cost. Under these constraints, clinical diagnosis based on only smear positivity using only Gram or Giemsa stain is likely to be much lower, as is evident from Table 1. Comparatively, the PCR technique can be easily added to the armamentarium of diagnostic methods in a microbiology laboratory. Additionally, the short time taken by the PCR test is a distinct advantage over the culture method. Hence, we strongly believe that the PCR based diagnostic assay, coupled with smear examination, will be very helpful and desirable for rapid diagnosis of *Acanthamoeba* keratitis and be confirmatory in clinically suspected cases with or without culture results.

The high clinical value of PCR in the diagnosis of *Acanthamoeba* keratitis has already been shown by Lehmann et al. (6), who found a sensitivity of 84%, which is similar to ours (87.5%), although they used clinical diagnosis as the gold standard as opposed to culture, which was the gold standard in this study. Evaluation of PCR for *Acanthamoeba* in consecutive cases of suspected microbial keratitis, as done in the present study, has further confirmed the applied value of molecular diagnosis in *Acanthamoeba* keratitis. A novel approach by Mathers et al. (7) of confirming confocal microscopy diagnosis of *Acanthamoeba* keratitis in contact lens wearers using PCR highlights the wide scope of utility of PCR assays. Based on their findings, the authors have speculated that *Acanthamoeba* may be responsible for a large percentage of cases that are commonly diagnosed as contact lens overwear. They have alluded to the possible association of *Acanthamoeba* with many forms of corneal epitheliopathy. Similar associations, which may or may not be related to contact lens wear, are expected to emerge in our setup with improved diagnosis using PCR test for *Acanthamoeba*. Such findings may lead to a revision of the incidence and prevalence data of the ocular or extraocular diseases caused by *Acanthamoeba* species in this part of the world.

Vodkin et al. (15) were the first to use PCR for the genus-specific detection of *Acanthamoeba*, using primer pair ACARNA.for1383 and ACARNA.rev1655, which amplifies 272 bp of

18S rDNA. This primer pair was also tested by Lehmann et al. (6) in their clinical study, along with a second 18S rDNA-based primer pair, P1GP.for2379 and P1GP.Rev2632, which amplifies a 253-bp amplicon. Analyzing complete 18S rDNA sequences of over 80 isolates of *Acanthamoeba*, Schroeder et al. (9) have shown that the above two primer pairs could also amplify rDNA of related amoebae, i.e., *Balamuthia* and *Hartmannella* spp. The primers used in our study (JDP1-JDP2) were designed by them from a large database of 18S rDNA sequences and were shown to be genus specific for *Acanthamoeba*. These primers had failed to amplify DNA from closely related amoebae and from several bacterial, fungal, and human DNAs. Although Schroeder et al. (9) used different PCR conditions for achieving high sensitivity and specificity in their study, in this study we have employed only one set of PCR conditions that provide both high sensitivity and specificity. Stothard et al. (13), using genus- and subgenus-specific oligonucleotide probes, have shown the specific identification of *Acanthamoeba* in both environmental and clinical samples. Therefore, in our opinion, it would be interesting and gainful to integrate such new PCR techniques in ocular microbiology laboratories dealing with large number of patients with microbial keratitis.

Inclusion of PCR for *Acanthamoeba* along with conventional methods of diagnosis of nonviral microbial keratitis is expected to improve the diagnosis of *Acanthamoeba* keratitis in ocular microbiology laboratories that have molecular biology facilities. While awareness regarding bacterial and fungal keratitis is relatively high in a majority of the eye hospitals in India, as reflected in several publications, information regarding *Acanthamoeba* keratitis is grossly inadequate. The reported incidence of *Acanthamoeba* keratitis in India varies from 1 to 3%, and the cases are predominantly in non-contact lens wearers (3, 10, 14). Through this report, we would like to emphasize that with appropriate tests a greater number of cases of *Acanthamoeba* keratitis can be differentiated from bacterial, fungal, or viral keratitis and treated appropriately before it is too late.

#### ACKNOWLEDGMENTS

We thank the Department of Biotechnology, Government of India, New Delhi, for the financial support to carry out the study.

We thank Thomas J. Byers, Ohio State University, Columbus, for 18S rDNA primer sequences.

#### REFERENCES

- Behzadbehbahani, A., P. E. Klapper, P. J. Valley, and G. M. Cleator. 1997. Detection of BK virus in urine by polymerase chain reaction: a comparison of DNA extraction methods. *J. Virol. Methods* 67:161-166.
- Chomczynski, P., K. Mackey, R. Drews, and W. Wilfinger. 1997. DNA Zol:

- a reagent for the rapid isolation of genomic DNA. *BioTechniques* **22**:550–553.
3. **Davamani, F., J. Gnanaselvam, K. Anandakannan, N. Sridhar, and T. Sundararaj.** 1998. Studies on the prevalence of *Acanthamoeba* keratitis in and around Chennai. *Ind. J. Med. Microbiol.* **16**:152–153.
  4. **Hugo, E. R., V. J. Stewart, R. J. Gast, and T. J. Byers.** 1992. Purification of amoeba mtDNA using the UNSET procedure, p. D7.1. *In* A. T. Soldo and J. J. Lee (ed.), *Protocols in protozoology*. Allen Press, Lawrence, Kans.
  5. **Kunimoto, D. Y., S. Sharma, P. Garg, U. Gopinathan, D. Miller, and G. N. Rao.** 2000. Corneal ulceration in the elderly in Hyderabad, south India. *Br. J. Ophthalmol.* **84**:54–59.
  6. **Lehmann, J. O., S. M. Green, N. Morlet, M. F. Keys, M. M. Matheson, J. K. G. Dart, J. I. McGill, and P. J. Watt.** 1998. Polymerase chain reaction analysis of corneal epithelial and tear samples in the diagnosis of *Acanthamoeba* keratitis. *Investig. Ophthalmol. Vis. Sci.* **39**:1261–1265.
  7. **Mathers, W. D., S. E. Nelson, J. L. Lane, M. E. Wilson, R. C. Allen, and R. Folberg.** 2000. Confirmation of confocal microscopy diagnosis of *Acanthamoeba* keratitis using polymerase chain reaction analysis. *Arch. Ophthalmol.* **118**:178–183.
  8. **Pitcher, D. G., N. A. Saunders, and R. J. Owen.** 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett. Appl. Microbiol.* **8**:151–156.
  9. **Schroeder, J. M., G. C. Booton, J. Hay, I. A. Niszl, D. V. Seal, M. B. Markus, P. A. Fuerst, and T. J. Byers.** 2001. Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of *Acanthamoeba* from humans with keratitis and from sewage sludge. *J. Clin. Microbiol.* **39**:1903–1911.
  10. **Sharma, S., P. Garg, and G. N. Rao.** 2000. Patient characteristics, diagnosis and treatment of non-contact lens related *Acanthamoeba* keratitis. *Br. J. Ophthalmol.* **84**:1103–1108.
  11. **Sharma, S., M. Srinivasan, and C. George.** 1990. *Acanthamoeba* keratitis in non-contact lens wearers. *Arch. Ophthalmol.* **108**:676–678.
  12. **Sharma, S., S. Athmanathan, M. A. U. Rasheed, P. Garg, and G. N. Rao.** 2001. Evaluation of immunoperoxidase staining technique in the diagnosis of *Acanthamoeba* keratitis. *Ind. J. Ophthalmol.* **49**:181–186.
  13. **Stothard, D. R., J. Hay, J. M. Schroeder-Diedrich, D. V. Seal, and T. J. Byers.** 1999. Fluorescent oligonucleotide probes for clinical and environmental detection of *Acanthamoeba* and the T4 18Sr RNA gene sequence type. *J. Clin. Microbiol.* **37**:2687–2693.
  14. **Srinivasan, M., C. A. Gonzales, C. George, V. Cevallos, J. M. Mascarenhas, B. Asokan, J. Wilkins, G. Smolin, and J. P. Whitcher.** 1997. Epidemiology and aetiological diagnosis of corneal ulceration in Madurai, south India. *Br. J. Ophthalmol.* **81**:965–971.
  15. **Vodkin, M. H., D. K. Howe, G. S. Visvesvara, and G. L. McLaughlin.** 1992. Identification of *Acanthamoeba* at the generic and specific levels using the polymerase chain reaction. *J. Protozool.* **39**:378–385.