

Prospective Comparison of Microbial Culture and Polymerase Chain Reaction in the Diagnosis of Corneal Ulcer

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- **PURPOSE:** To compare polymerase chain reaction (PCR) to microbial culture for the detection and identification of bacterial and fungal pathogens in microbial keratitis.
- **DESIGN:** Prospective cohort study.
- **METHODS:** A total of 108 consecutive corneal ulcers were cultured and analyzed by PCR using pan-bacterial and pan-fungal primers. PCR products were cloned, sequenced, and compared to culture results using standard bioinformatics tools.
- **RESULTS:** Of the 108 samples, 56 were culture-positive, 25 for bacteria and 31 for fungi; 52 were culture-negative. After eliminating false-positive PCR products, 94 of 108 were positive by PCR, 37 for bacteria and 57 for fungi. Nineteen of 25 bacterial culture-positive samples were positive by PCR, and 29 of 31 samples culture-positive for fungi were positive by PCR. The majority of sequenced PCR products matched the positive culture results. Of the 52 culture-negative samples, 46 (88%) yielded pathogen deoxyribonucleic acid (DNA) PCR products, 18 bacterial and 28 fungal. These represented a variety of species, including at least three novel previously uncultured microbes.
- **CONCLUSIONS:** PCR detects microbial DNA in the majority of bacterial and fungal corneal ulcers, and identifies potentially pathogenic organisms in a high proportion of culture-negative cases. Yield and concordance with culture are higher for fungal than bacterial ulcers. Practical use of the technique is limited by artefactual amplification of nonpathogenic organisms. PCR may be used as an adjunct to culture to identify potential pathogens in microbial keratitis. (Am J Oph-

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MICROBIAL KERATITIS IS A MAJOR CAUSE OF blindness, particularly in the developing world.^{1–3} In Madurai, India, the incidence of corneal ulceration is 113 per 100,000 person-years, approximately 10 times that found in the Olmstead County, Minnesota population in the United States.^{2,4,5} In other parts of the developing world, the annual incidence of corneal ulceration has reached epidemic proportions. A prospective, population-based study in Nepal found an annual incidence of 799 per 100,000 person-years.⁶ The number of corneal ulcers occurring annually in the world likely approaches two million.^{7,8}

The distinction between bacterial and fungal etiology is essential to management. Currently, the standard for diagnosing and characterizing microbial keratitis is culture. Culture results are highly specific but have suboptimal sensitivity, generally yielding results in fewer than 70% of cases.² Microbial culture is also slow, with growth and identification taking approximately three to five days for bacteria and approximately five to seven days for fungi.

The polymerase chain reaction (PCR) is a highly sensitive and rapid technique for amplifying analytic quantities of deoxyribonucleic acid (DNA) from infinitesimal starting quantities. When applied to the detection of pathogen DNA, the technique can be used to rapidly identify the presence of specific organisms.⁹ PCR has been widely applied to the diagnosis of viral uveitis, infectious endophthalmitis, and parasitic eye disease, as well as viral keratitis,¹⁰ but application to microbial keratitis has been more limited.^{11–16} In the present study, we sought to prospectively compare culture and microbial PCR results in a series of patients presenting with a corneal ulcer.

METHODS

- **SAMPLE ACQUISITION:** All participants were recruited from the Aravind Eye Hospital in Madurai, India. Patient inclusion criteria were: presence of a corneal ulcer with an overlying epithelial defect >1 mm at its greatest width; some portion of the infiltrate covering the central third of

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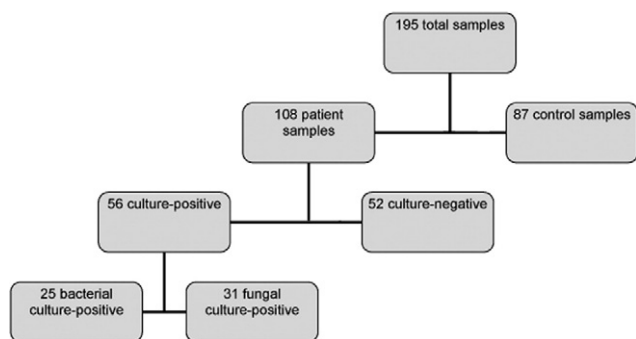


FIGURE 1. Culture results from collected corneal ulcers.

the cornea; and ability to provide appropriate consent. Exclusion criteria were: bilateral corneal ulcers; corneal ulcers of viral or parasitic origin (as suggested by history and examination findings); presence of endophthalmitis; and inability to give consent. A total of 108 patients met inclusion criteria.

Corneal scrapings for culture were obtained with a flame-sterilized Kimura spatula, after instillation of topical anesthetic (e.g., 0.5% tetracaine). The spatula was used to scrape from the leading edge to the base of the ulcer. The spatula was flame-sterilized between each sample. Two scrapings were smeared directly onto two separate glass microscope slides for Gram stain and potassium hydroxide (KOH) wet mount. Three further scrapings were directly inoculated onto sheep's blood agar, chocolate agar, and potato dextrose agar or Sabouraud's agar for bacterial and fungal culture. After culture samples had been obtained, a sterile Dacron swab was used to obtain a corneal scrape from the base and leading edge of the corneal ulcer. The swab was then placed into a sterile microcentrifuge tube and capped. For 87 of the 108 patients, "air" swabs, exposed only to the air surrounding the patient and handled identically to the patient samples, were obtained as controls for local environment and placed in sterile microcentrifuge tubes. Tubes were stored at -80°C until they were shipped on dry ice to Washington University, St Louis, Missouri, for processing. Swabs were immediately transferred on receipt to -80°C storage and were not allowed to thaw before processing. DNA from all samples was extracted within one month of receipt.

Culture growth was read at 48 hours for bacterial growth and seven days for fungal growth. If positive, the colony was further analyzed by standard biochemical tests until a more specific species was identified. PCR was performed without knowledge of culture results.

• **DEOXYRIBONUCLEIC ACID EXTRACTION, POLYMERASE CHAIN REACTION, AND SEQUENCING:** Briefly, DNA was extracted from each swab following treatment with lysozyme and zymolase to break down microbial cell walls. PCR was performed using standard, validated 16S (bacterial¹⁷) and 18S (fungal¹⁸) primer sets. Methoxypso-

ralen was used to minimize carry-over contamination.¹⁹ Clone libraries were generated from positive PCR primers and subjected to DNA sequencing. Complete details of PCR, cloning, and sequencing protocols are available online (see Supplemental METHODS SECTION available at AJO.com).

RESULTS

• **CULTURE RESULTS:** There were a total of 195 swabs used in this study, 108 patient samples and 87 air swab controls ("air swabs"). Of the 108 patient samples, 56 were culture-positive (25 bacterial, 31 fungal) and 52 were culture-negative (Figure 1). The most frequent bacterial pathogens isolated from corneal ulcers were *Streptococcus pneumoniae* (18 cases) and *Pseudomonas* species (eight cases). Three cases were positive for *Nocardia*, and one was positive for *Bacillus*. Two cases were polymicrobial, one for *S. pneumoniae* and *Nocardia*, and one for *S. pneumoniae* and *Fusarium*. For fungal cultures, 20 were positive for *Fusarium*, five were positive for *Aspergillus*, and one each was positive for *Botryodiplodia* and *Curvularia*.

• **POLYMERASE CHAIN REACTION RESULTS:** Of the 87 "air" control swabs, 45 samples were PCR-positive and 42 were PCR-negative. All 45 PCR-positive control samples were cloned and sequenced. Of these, four (4.59%) yielded sequences for pathogens associated with fungal keratitis (*Fusarium* and *Aspergillus*, two isolates each). The remaining 41 products showed sequences for one of the following microbial genera: *Ralstonia*, *Oerskovia*, *Leclercia* (bacteria typically found in air, soil, or moist environments that have very rarely been associated with opportunistic human disease,²⁰⁻³⁰ 33 sequences total), or *Saccharomyces* (yeast that very rarely has been associated with ocular disease,³¹ eight sequences total). The source of these contaminating sequences appeared to be the sample swabs from the clinical site, as control experiments subjecting sterile Dacron swabs within the processing laboratory to the same extraction and amplification conditions as the patient samples resulted in no bacterial or fungal PCR products. For purposes of determination of pathogen DNA identity, PCR-derived clones yielding sequences for *Ralstonia*, *Oerskovia*, *Leclercia*, or *Saccharomyces* were considered to represent environmental contaminant DNA.

Of the 108 samples derived from corneal ulcers, 96 yielded PCR products with either bacterial primers, fungal primers, or both (Figure 2). Complementary deoxyribonucleic acid (cDNA) libraries were made from each PCR product and 12 colonies picked from each. Ninety-four of the 96 samples (97.9%) resulted in sequence for suspected pathogens (58 fungal and 36 bacterial) in the 12 colonies picked from their respective libraries, while two samples (2.1%) resulted in only contaminant sequences. In addition to these two samples, 50 samples (53.6%) with clones

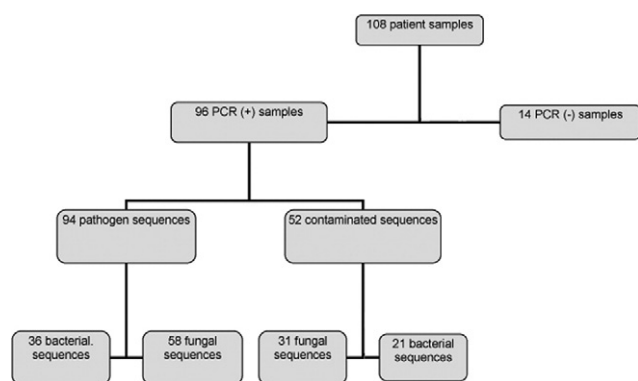


FIGURE 2. Polymerase chain reaction results from collected corneal ulcers.

for potential pathogens also resulted in at least one clone representing a potential contaminant (total contaminant sequences: 31 fungal and 21 bacterial).

Of the 31 corneal ulcers culture-positive for fungi, 29 were PCR-positive for fungi (Table 1). The two culture-positive, PCR-negative ulcers both grew *Aspergillus*. Of the 58 samples positive by fungal PCR, 29 were culture-positive and 29 were culture-negative. Of the 29 culture-positive, PCR-positive samples, DNA sequence yielded the same organism as culture in 26 (89.6%) (Table 2). The three discrepancies between sequencing and PCR were: one culture-positive for *Botryodiplodia* and PCR-positive for *Fusarium*; one culture-positive for *Aspergillus* and PCR-positive for *Fusarium*, and one culture-positive for *Aspergillus* and PCR-positive for *Alternaria*. Of the 29 culture-negative, PCR-positive samples, sequence analysis revealed a potential pathogen in all cases. Seventeen of 29 were positive for either *Fusarium* (10), *Aspergillus* (3), or both *Fusarium* and *Aspergillus* (4). The 12 remaining PCR products were positive for *Sordaria*, *Pythium*, *Botryosphaeria*, *Cladosporium*, *Calcarisporium*, or *Penicillium*. One sample (clone 102) did not perfectly match any fungus in the National Center for Biotechnology Information (NCBI) database, and so was submitted as a novel sequence (NCBI GenBank accession no. EU529708). BLAST analysis of the 420 bp of high-quality sequence showed closest match (99% nucleotide identity) an uncultured blastidiomycete fungus (NCBI GenBank accession no. AF530542).

Of the 25 samples culture-positive for bacteria, 19 were PCR-positive, while six samples were culture-positive and PCR-negative. Three of these six samples were positive for *Nocardia* by culture and negative by PCR, while three were culture-positive for *Streptococcus*. PCR yielded the same organism as culture in 12 of the 19 (63.2%) (Table 3). Of the 11 ulcers culture-positive for *S. pneumoniae* and PCR-positive, eight sequences matched *S. pneumoniae*, and one each matched *Corynebacterium*, *Enterococcus*, and *Simonsiella*. Of the five ulcers culture-positive for *Pseudomonas aeruginosa*, three were PCR-positive for *Pseudomo-*

nas, while one each was positive for *Streptococcus* and *Neisseria*. One ulcer culture-positive for *Staphylococcus epidermidis* was PCR-positive for *Streptococcus*. Finally, one ulcer culture-positive for *Bacillus* was PCR-positive for *Fusarium*, the only sample in this study that was cultured for bacteria but yielded a positive pathogen PCR result for fungus.

Of the 36 samples PCR-positive for bacteria, 19 were culture-positive and 17 were culture-negative. Of the 17 culture-negative, PCR-positive samples, sequence analysis revealed potentially pathogenic bacterial DNA in all cases. The most commonly identified sequences were for *Corynebacteria* (4), *Streptococcus* (3), or *Pseudomonas* (2). Six culture-negative samples were positive for other organisms known to cause microbial keratitis, including *Haemophilus influenza* and *Moraxella*, *Salmonella*, and *Simonsiella* species. The remaining two culture-negative, PCR-positive sequences were most similar to uncultured bacteria. We have submitted these sequences to NCBI GenBank. Clone 790 (GenBank accession no. EU529710) yielded 600 bp of high-quality sequence from the 16S gene, and was 98% homologous to an uncultured *Sphingomonas* (NCBI GenBank accession no. EF662327). Clone 258 (GenBank accession no. EU529709) yielded 540 bp of high-quality sequence, and was a 97% match with an uncultured *Neisserium* clone (NCBI GenBank accession no. DQ847448).

DISCUSSION

MICROBIAL CULTURE REMAINS THE GOLD STANDARD FOR identification of pathogens causing corneal ulcers. While it is unclear whether ulcers treated on the basis of culture results have better outcomes than those treated empirically with broad-spectrum antibiotics,³² it does appear that identification of causative organisms has substantial prognostic import.³³ Culture results are also critical to tailoring therapy in patients who fail empiric therapy, as occurs in approximately 10% of cases.³⁴ The importance of correct identification of pathogen is increased in parts of the world where fungal keratitis is common, as choice of correct antimicrobial requires distinguishing fungal from bacterial etiology. The appearance of the ulceration is unreliable in distinguishing fungal from bacterial ulcer.³⁵

Unfortunately, microbial culture is a relatively insensitive diagnostic test, with growth seen in only about 50% to 70% of cases.³⁶ The PCR is a powerful technique for amplifying infinitesimal quantities of nucleic acids for further analysis. PCR is an extremely sensitive technique, able to detect single copies of pathogen DNA in complex mixtures. PCR has been successfully applied to the diagnosis of many ocular conditions, including viral retinitis,³⁷ viral and protozoal keratitis,^{38,39} and infectious endophthalmitis.⁴⁰ The availability of DNA primer sets that effectively recognize all bacteria or all fungi (i.e., pan-bacterial or pan-fungal) suggests this technique may have

TABLE 1. Comparison of Microbial Culture and Polymerase Chain Reaction Results for Corneal Ulcer

	Bacterial			Fungal		
	PCR-Positive	PCR-Negative	Total	PCR-Positive	PCR-Negative	Total
Culture-Positive	19	6	25	29	2	31
Culture-Negative	17	6	23	29	6	35
Total	36	12	48	58	8	66

PCR = polymerase chain reaction.

utility for diagnosis of microbial keratitis. Several previous studies have applied either pan-bacterial or pan-fungal PCR for microbial keratitis in small numbers of patients. Knox and associates studied 10 patients with culture-positive microbial keratitis and 17 with culture-negative keratitis.⁴¹ Eight of 10 patients who were culture-positive in this study were PCR-positive, with complete concordance between culture and PCR sequencing results. None of 17 other keratitis patients (with forms of keratitis thought to be nonbacterial) were positive for bacterial products. This study differed from the present study in that the setting for acquisition was a tertiary care facility, and inclusion criteria required bacterial growth from the corneal ulcer. Rudolph and associates¹¹ studied four patients with severe infectious keratitis and either negative cultures or culture results incompatible with the clinical course. Direct sequencing of PCR products revealed unusual species in several cases, including *Abiotrophia defectiva*, *Stenotrophomonas maltophilia*, and *Porphyromonas gingivalis*. Schabereiter-Gurtner and associates¹⁵ analyzed the total bacterial community from one patient with a recalcitrant corneal ulcer resistant to many months of topical antibiotic therapy, using PCR and denaturing gradient gel electrophoresis.¹³ The authors found massive polymicrobial infection with at least 17 species present. Gaudio and associates¹⁶ performed nested pan-fungal PCR on scrapings of 30 patients with presumed infectious keratitis, of whom 15 were positive by culture for fungi. Only one culture-positive ulcer in this study was PCR-negative, while seven ulcers were culture-negative but PCR-positive. Amplified products were not sequenced so concordance of speciation could not be assessed. Kumar and associates¹² have studied the use of PCR in detecting fungal pathogens in keratitis. In this study, samples from four patients with mycotic keratitis were studied, along with other samples obtained directly from fungal cultures. The authors found the PCR combined with single-stranded conformational analysis allowed rapid and precise identification of unusual mycotic pathogens. Subsequent work from this group has confirmed the utility of this approach.⁴² To date, however, no study has prospectively compared ensemble bacterial and fungal PCR analysis to culture.

In the present study, microbial culture yielded an organism in 56% of cases. This is similar to the overall 63% bacterial and fungal culture-positive rate described in a

consecutive series of 3,298 eyes with microbial keratitis from another institution in India.⁴³ Consistent with previous studies in India, the majority of culture-positive ulcers in this population were fungal.⁴³⁻⁴⁶ After accounting for contaminating DNA sequences, the majority of culture-positive ulcers were also PCR-positive. PCR appeared to have a higher yield in fungal ulcers than in bacterial ulcers, with 29 of 31 fungal culture-positive ulcers (94%) yielding a positive PCR product vs 19 of 25 bacterial culture-positive ulcers (76%). Half of the culture-positive, PCR-negative cases grew *Nocardia*, suggesting reduced sensitivity for detection of this genus by PCR. Previous studies have demonstrated, however, that *Nocardia* rDNA can be amplified with universal 16S primers,⁴⁷ and one culture-positive case for *Nocardia* was detected by PCR in the present study. It is possible that with different DNA extraction technology, yield for detection of *Nocardia* could have been improved. Importantly, only one ulcer of the 108 studied yielded a bacterium on culture and a fungal pathogen on PCR. This strongly suggests that PCR may be used to distinguish bacterial from fungal pathogenesis.

Concordance of DNA sequencing results with cultured organism was excellent for fungal species, with 89% agreement (26/29). PCR detected polymicrobial fungal infection in two cases where only a single fungus was recovered by culture. Concordance was lower for bacterial ulcers, with 63% agreement (12/19). The lower concordance rate with bacterial ulcers may be attributable to detection of normal ocular surface biota by PCR. Fungi are rarely cultured as normal commensal organisms of the ocular surface,⁴⁸ while several potentially pathogenic bacteria such as *Corynebacteria* and *S. epidermidis* are commonly found in the normal conjunctival flora.⁴⁹ Thus, the discrepant case in which *S. pneumoniae* was cultured while *Corynebacterium* was detected by PCR may represent PCR amplification of the commensal organism. The converse case, in which *S. epidermidis* was identified by culture but *S. pneumoniae* was found by PCR, could possibly represent detection of the commensal organism by culture. The appropriate interpretation of discrepant culture and PCR results is presently unclear; however, in the setting of active corneal ulceration, it may be prudent to consider culture recovery or PCR identification of highly virulent organisms such as *Pseudomonas* or *Neisseria* as evidence of infection with those microbes.

TABLE 2. Comparison of Culture and Polymerase Chain Reaction Results for Fungal Corneal Ulcers

Culture Results	PCR Results											No PCR Result
	Fusarium	Aspergillus	Botryodiplodia	Curvularia	Sordaria	Alternaria	Fusarium and Aspergillus	Penicillium	Phythium	Cladosporium	Uncultured Fungus	
Fusarium	19	—	—	—	—	—	1	—	—	—	—	0
Aspergillus	1	4	—	—	—	1	1	—	—	—	—	2
Botryodiplodia	1	—	—	—	—	—	—	—	—	—	—	0
Curvularia	—	—	—	1	—	—	—	—	—	—	—	0
Negative	0	3	2	—	3	—	4	1	3	1	1	6

PCR = polymerase chain reaction.

TABLE 3. Comparison of Culture and Polymerase Chain Reaction Results for Bacterial Corneal Ulcers

Culture Results	PCR Results												No PCR Results	
	<i>Streptococcus pneumoniae</i>	<i>Pseudomonas aeruginosa</i>	<i>Nocardia and Fusarium</i>	<i>Staphylococcus epidermidis</i>	Corynebacterium	Enterococcus	<i>H. influenzae</i>	Moraxella	Neisseria	Simonsiella	Salmonella	Fusarium		Uncultured
<i>S. pneumoniae</i>	8	—	—	—	1	1	—	—	—	1	—	—	—	3
<i>P. aeruginosa</i>	1	3	—	—	—	—	—	—	1	—	—	—	—	—
Nocardia	—	—	1	—	—	—	—	—	—	—	—	—	—	3
<i>S. epidermidis</i>	1	—	—	—	—	—	—	—	—	—	—	—	—	—
Bacillus	—	—	—	—	—	—	—	—	—	—	1	—	—	—
Negative	3	2	—	1	4	1	1	1	1	—	—	1	2	6

PCR = polymerase chain reaction.

TABLE 4. Summary of Polymerase Chain Reaction-Positive Results for Culture-Negative Swabs From Corneal Ulcers

	PCR (-)	Sequence-Positive: Bacterial	Sequence-Positive: Fungal	Total
Culture-negative swabs	6	17	29	52
# of each sequence		4 <i>Corynebacterium</i>	10 <i>Fusarium</i>	46
		3 <i>S. pneumo</i>	4 <i>Fusarium</i> and <i>Aspergillus</i>	
		2 <i>Pseudomonas</i>	3 <i>Aspergillus</i>	
		2 Uncultured	3 <i>Sordaria</i>	
		1 <i>S. epidermidis</i>	3 <i>Phythium</i>	
		1 <i>H. influenza</i>	2 <i>Botryosphaeria</i>	
		1 <i>Neisseria</i>	1 <i>Cladosporium</i>	
		1 <i>Moraxella</i>	1 <i>Calcarisporium</i>	
		1 <i>Salmonella</i>	1 <i>Penicillium</i>	
		1 <i>Simonsiella</i>	1 Uncultured	

PCR = polymerase chain reaction.

Of the 52 culture-negative samples in the present study, 46 (88%) yielded PCR results suggestive of potential pathogens (Table 4). The proportions of bacterial (39%) and fungal (61%) pathogens mirrored the prevalence of bacterial and fungal pathogens found in the culture-positive group (45% and 55%, respectively). The majority of organisms amplified by PCR in these cases were known pathogens. For bacteria, eight of 15 of the identified pathogens in these cases were *S. pneumoniae*, *P. aeruginosa*, *H. influenza*, *Moraxella*, or *Neisseria*, all well-recognized bacterial causes of microbial keratitis. For the fungal cases, 13 of 28 PCR products in culture-negative cases corresponded to *Fusarium* and *Aspergillus* species. *Curvularia*, *Alternaria*, *Penicillium*, and *Cladosporium* have all been identified as pathogens in previous studies of corneal ulceration in Madras, India.⁵⁰ Two cases in the current series were PCR-positive for *Botryodiplodia*. This has been identified as a cause of corneal ulceration in a single case report previously.⁵¹ *Phythium* species have previously been identified with recalcitrant cases of microbial keratitis, particularly in Thailand, where 10 cases were reported occurring over a 10-year period.⁵²⁻⁵⁶ The pathogenesis of the PCR-negative, culture-negative cases is unclear. Our technique may not have had sufficient sensitivity to detect low levels of viable but nonculturable bacterial pathogens. Other organisms known to cause microbial keratitis, such as *Acanthamoeba* or microsporidia, would not be detected by pan-bacterial and pan-fungal primer sets.

Interestingly, PCR positivity for a number of organisms not previously associated with microbial keratitis was observed in this study. One corneal ulcer was positive for *Simonsiella*. This gram-negative filamentous bacterium has previously been associated with erosive oral ulcers.⁵⁷ Corneal ulcers have not been previously associated with *Sordaria* or *Calcarisporium* species. Additionally, the study found sequence from one previously uncultured fungus and two previously uncultured fungi (whose DNA sequences are

found in the NCBI database as a result of molecular “biome mining”). Together, these cases and those previously associated only with a single previous case report of corneal ulceration represented nine of the 46 culture-negative, PCR-positive ulcers in the study. Importantly, these organisms were not detected by PCR in cases where known pathogens were identified by PCR. While the presence of microbial DNA as detected by PCR does not fulfill Koch’s postulates for demonstrating pathogenicity, this finding does suggest the hypothesis that the etiology of a substantial fraction of culture-negative corneal ulcers (perhaps as high as 20% in the present study) may be attributable to unusual or novel micro-organisms. In previous large studies of the etiology of corneal ulcers in India, significant numbers (65/1132) of fungal cultures yielded organisms that could not be identified.⁴³ It is possible that some of these unidentifiable fungi may correspond to the novel or unusual fungi identified by PCR. These results are consistent with studies in bacterial endophthalmitis, in which the majority of culture-negative cases yielded bacterial sequences following PCR, with about half (8) of these culture-negative, PCR-positive cases associated with novel bacteria.⁵⁸ Further molecular analysis with acquisition of additional sequence information may improve our understanding and ability to detect these potential pathogens in future studies.

The results of the present study are qualitatively similar to those of other prospective studies comparing broad-spectrum microbial PCR to culture for infectious disease. Welinder-Olsson and associates⁵⁹ prospectively compared 16S ribosomal PCR to culture for the diagnosis of bacterial meningitis from cerebrospinal fluid material. Of 65 samples positive by either test, 14 were culture-positive but PCR-negative, 25 patients were positive by both PCR and culture, and 26 were positive by PCR only. Similar to the present study, the majority of PCR-positive, culture-negative cases showed positivity for known pathogens such as *Neisseria meningitidis* and *S. pneumoniae*. The authors also recovered sequences by PCR that were felt to be commen-

sal contaminants of the spinal tapping procedure (coagulase-negative *Staphylococcus* species) and novel organisms of unclear pathogenic significance (*Fusobacteria* and *Granulicatella*). Dempsey and associates⁶⁰ used 16S ribosomal amplification to investigate the etiology of clinically infected prosthetic hip joints in 10 patients. Nine bacterial species were identified by culture but 30 species were identified by PCR, including at least 10 previously uncultured bacteria and several bacteria not previously associated with human disease. Mardh and associates compared the use of fungal PCR to culture in the diagnosis of vulvovaginal infection.⁶¹ Of 73 patients tested, 43.8% were positive by both PCR and culture, 20.5% were positive by culture only, and 17.8% were positive by PCR only, while 23.3% were negative by both tests. Of the cases that were positive by PCR and culture, 12.5% were discordant for sequence identification. Similar to the results of the present investigation, these studies suggest that pan-bacterial and pan-fungal PCR are generally as sensitive as or more sensitive than culture for the detection of micro-organisms in infectious disease; that PCR may identify many organisms not routinely seen in culture; and that PCR and culture may be discrepant in species identification.

Enthusiasm for use of PCR in diagnosis for corneal ulcers must be tempered by difficulties encountered in this study. We saw a very high rate of false positives for apparently nonpathogenic organisms, *Ralstonia*, *Oerskovia*, *Leclercia*, and *Saccharomyces*. *Oerskovia*³⁰ and *Saccharomyces*³¹ have very rarely been associated with corneal ulceration. *Ralstonia*⁶² and *Leclercia*^{22,63} have been very rarely associated with any form of human infection. The identification of these organisms in 45 of 87 control swabs and 56 of 94 patient samples strongly suggests they are contaminants. False-positive results for pan-bacterial and pan-fungal PCR are a well-recognized problem with this technique.⁶⁴ As we could not detect these organisms in sample swabs originating in the PCR facility, and as we took substantial measures to prevent cross-contamination of samples with foreign DNA,¹⁹ it is likely these sequences originated at the time of sample acquisition in India. Airborne contamination of PCR reactions has been well described.⁶⁵ Significant aerosol contamination with bacteria and fungi have been identified in many settings. *Oerskovia*, for example, has been detected in cultures derived from air samples taken from swine farms,⁶⁶ and

Ralstonia have been found with high frequency in air samples taken from the Washington, DC subway system.⁶⁷ *Saccharomyces cerevisiae* has been reported as an environmental contaminant in fungal PCR reactions in other studies.⁶⁸ *S. cerevisiae* has also been identified as a contaminant in some zymolase preparations;⁶⁹ it is possible a contaminated lot was used on patient samples but not in the controls run within the laboratory. These false positives unfortunately prevented rapid discrimination of fungal from bacterial infection, and necessitated sequencing of multiple clones for identification of pathogenic species. The presence of contaminating microbial DNA thus prevents application of more efficient means of identification such as single-stranded polymorphism testing,⁴² denaturing gradient gel electrophoresis,^{13,70} or direct sequencing of DNA products.¹¹ Turnaround time for PCR, cloning, and sequencing was on the order of five to seven days, making the technique slower than traditional sequencing and bacterial culture and on par with fungal cultures. The extent to which similar contamination would occur in other clinical settings remains to be determined. Finally, PCR for genomic DNA cannot distinguish between viable and dead micro-organisms. The effect of analyzing RNA expression (via reverse-transcription PCR) along with genomic DNA expression on sensitivity and specificity of pathogen detection in microbial keratitis has not been systematically studied to date.

Despite these limitations, PCR may provide useful information on the pathogens associated with corneal ulcers. As there were substantial numbers of culture-positive, PCR-negative ulcers in the present study, it is clear that PCR is complementary to culture and cannot replace traditional culturing. We have found that PCR reliably distinguishes bacterial from fungal pathogen. This may be particularly useful in cases where KOH testing of scrape material is falsely negative, as occurs in ~40% of fungal ulcers presenting early in clinical course.⁷¹ We also find that PCR combined with DNA sequencing can identify the presence of known pathogen DNA in the majority of culture-negative cases. The significance of novel organisms detected by PCR in culture-negative ulcers remains to be determined. Further development of this technique is warranted, with particular attention to reducing false-positive results from contaminating DNA.

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SUPPLEMENTAL METHODS

• **DEOXYRIBONUCLEIC ACID EXTRACTION:** Each swab was sterilely transferred into a new sterile Eppendorf 1.5-ml lock-top tube. Then, 300 μ l of lysozyme-containing solution (6 mg lysozyme, 20 mM Tris-HCl pH 8.0, 2 mM ethylenediaminetetraacetic acid (EDTA), 1.2% Triton X-100) was added to each lock-top tube containing the Dacron swab to break down the cell walls of gram-positive bacteria. The tube was placed into a 37 C incubation for one hour and vortexed every 10 minutes for 10 seconds at a time. One hundred and fifty microliters of this solution was reserved in another sterile 1.5-ml microcentrifuge tube while 300 μ l of zymolase solution (50 mM Tris-HCl pH 7.5, 10 mM EDTA, 0.2% b-mercaptoethanol, three units zymolase) was added. The tube was again incubated at 37 C for one hour, vortexing every 10 minutes for 10 seconds. The previously reserved 150 μ L from the lysozyme incubation was added to the tube containing the swab and the zymolase solution. The Qiagen QIAamp DNA Micro Kit (Qiagen, San Diego, California, USA) was used to extract deoxyribonucleic acid (DNA), following the manufacturer's protocol, including the use of carrier ribonucleic acid (RNA). The final elution was performed with 50 μ l of the EA buffer (provided by Qiagen). Five microliters was taken from each sample to undergo polymerase chain reaction (PCR) analysis.

• **POLYMERASE CHAIN REACTION:** Bacterial PCR was performed with 16S rDNA universal primers (27f and 907r) (5'-AGA GTT TGA TCC TGG CTC AG-3' and 5'-CCC CGT CAA TTC ATT TGA GTT T-3').¹⁷ Fungal PCR was performed with 18S universal rDNA primers (18S forward and 18S reverse) (5-ATT GGA GGG CAA GTC TG-3' and 5'-CCG ATC CCT AGT CGG CAT AG-3').¹⁸ For each reaction, 5 μ l of the eluted sample DNA was combined with 5 μ l of 10X PCR buffer (500 mM potassium chloride; 100 mM Tris hydrochloride [pH 9.0 at 25 C]; 1.0% Triton X-100); 3 μ l of 25-mM magnesium chloride; 1 μ l of 0.2-mM each dinucleotide triphosphates (dNTP), 25 pmol of each primer; 0.5 μ l of 2.5 mg/ml 8-methoxypsoralen; and 0.25 U of Taq DNA polymerase (Invitrogen, Carlsbad, California, USA) in a total volume of 50 μ l. All PCR reactions were performed in a dedicated PCR hood (CleanSpot PCR workstation; Coy Laboratory Products, Grass Lake, Michigan, USA). Separate positive control PCR for *E. coli* and control *S. cerevisiae* were performed with each PCR batch. To mini-

mize possibly carryover contamination, the 10X buffer, 25 mM MgCl₂, and Taq polymerase were pretreated with ultraviolet (UV) light (254 nm) for four minutes to activate 8-methoxypsoralen to bind contaminating DNA.¹⁹ After the four-minute treatment, the dNTP mixture and the pair of forward and reverse primers were added to complete the master mix. Each sample was amplified in a 200- μ l thin-walled tube in an automated thermocycler with heated lid (MasterCycler gradient, Eppendorf). Cycling conditions for bacterial primers were: an initial five-minute denaturation at 95 C, followed by 40 cycles of one-minute denaturation at 95 C, one-minute annealing at 54.5 C, and one-minute extension at 72 C. Final extension step was five minutes at 72 C. Cycling conditions for fungal primers were: an initial five-minute denaturation at 95 C, followed by 45 cycles of one-minute denaturation at 95 C, one-minute annealing at 54.5 C, and one-minute extension at 72 C. Final extension step was five minutes at 72 C. All amplified DNA was detected by agarose gel electrophoresis visualized with ethidium bromide.

• **SEQUENCING:** 16S and 18S rDNA clone libraries were generated in pCR2.1 TOPO from the bacterial and fungal PCR products, respectively, by topoisomerase-mediated cloning following the manufacturer's protocol (Invitrogen). Libraries were transformed into *E. coli* TOP 10 chemically competent cells. The cloning transformation was plated on X-gal-containing agar plates. Twelve white (insert-containing) colonies for each PCR product were picked and incubated in a shaking incubator at 37 C for 20 hours in 2 ml of Magnificent Broth (MacConnell Research, San Diego, California, USA). The plasmid DNA was extracted using a MacConnell Miniprep 96 automated miniprep device. All mini-prep DNA samples underwent an EcoRI restriction digestion to verify insert presence. The digest samples were run in an ethidium bromide-stained gel to identify samples containing appropriately-sized inserts (890 bp for bacterial, 520 bp for fungal), which were sequenced using M13 forward sequencing primers with the ABI Big Dye system (Applied Biosystems, Foster City, California, USA). Presence of chimeric sequences was excluded using the Check Chimera program (<http://rdp8.cme.msu.edu/cgis/chimera.cgi?su=SSU>). Sequences were BLAST searched against the National Library of Medicine National Center for Biotechnology Information nonredundant (nr) database (<http://www.ncbi.nlm.nih.gov/BLAST/>).