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16S Ribosomal RNA PCR Versus Conventional Diagnostic Culture in the Investigation of Suspected Bacterial Keratitis

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Purpose: The purpose of this study was to compare conventional diagnostic culture (CDC) to 16S ribosomal RNA polymerase chain reaction (PCR) analysis for diagnosing bacterial keratitis.

Methods: Samples collected from 100 consecutive patients presenting to the Royal Liverpool University Hospital with bacterial keratitis were processed using CDC and 16S PCR analysis.

Results: The overall detection rate using both methods was 36%. Of these, 72.2% (26/36) were detected by PCR and 63.9% (23/36) isolated by CDC ($P = 0.62$). Using a combination of both PCR and CDC increased the detection rate for pathogenic bacteria by 13% compared to using CDC alone ($P = 0.04$). In CDC negative samples, 16S PCR identified more pathogens than CDC in 16S PCR negative samples. Neither order of sample collection nor prior antimicrobial use affected the detection rate.

Conclusions: 16S rRNA gene PCR performed in addition to CDC on corneal samples from patients with clinically suspected bacterial keratitis led to additional pathogen detection.

Translational Relevance: 16S rRNA gene PCR should be developed to become an additional part of clinical service for patients with bacterial keratitis rather than used in isolation.

Introduction

Bacterial keratitis is an ophthalmological emergency that can lead to sight threatening complications, such as corneal scarring, perforation, endophthalmitis, and ultimately blindness.[1] Improving outcomes depends on rapidly identifying the causative microorganism.

Currently, the likely causative microorganism is only isolated in 30% to 60% of cases using traditional scraping methods and standard conventional diagnostic culture (CDC), with results typically taking up to 3 days to become available to the clinician.[1–3] This may explain the reluctance of some ophthalmologists to perform a corneal scrape to reach a microbiological diagnosis. For example, McDonnell et al. found that 49% of ophthalmologists treated corneal ulcers empirically without attempting to identify the causative organism.[4] It is evident that improvements are required in the detection and diagnosis of the causative bacteria in cases of suspected bacterial keratitis.

These limitations have led to the identification of more sensitive, rapid processing methods. The 16S ribosomal DNA (16S RNA) gene is present in all bacteria and consists of highly conserved regions of nucleotide sequences, interspersed with 9 variable regions that are genus or species specific. Broad range polymerase chain reaction (PCR) primers that target the conserved regions can amplify the variable regions, which following sequencing and comparison to known sequences in a stored database will identify the genus or species of bacteria present in a sample.[5–8] Broad range bacterial PCR is especially advantageous in detecting slow-growing bacteria or those bacteria that are

traditionally difficult to cultivate.[9–11] These PCR techniques increase the potential to detect microorganisms but conversely may make it difficult for the treating clinician to interpret which of the identified organisms are causative.

In order to improve the microbiological diagnosis of a keratitis, different PCR techniques have been evaluated, but with varying concordance between culture and PCR results.[12–19] These studies largely comprised small numbers with different sampling techniques (scrape versus swab) and none considered the effect of sampling order on detection rates.

In this study, we compared the sensitivity of 16S rRNA gene PCR to CDC for the diagnosis of bacterial keratitis by controlling for the sampling technique, order of sampling, and taking into account the detection of potential contaminating species.

Methods

Consecutive patients with clinically suspected bacterial keratitis to the Royal Liverpool Hospital were recruited at presentation until 100 patients had been recruited. This sample size was determined on a difference between CDC and PCR of 20%, a type I error (α) of 0.05, and a power of 0.8. Three corneal scrapes were collected from the edge of the corneal ulcer of each patient, as previously described.[20] The first scrape was smeared onto a glass slide for Gram staining, the second was placed into bovine heart infusion (BHI) broth for CDC, and the third was transferred into a sterile tube for 16S rRNA gene PCR analysis. The order of sampling was: Gram stain, CDC, and PCR for the first 50 samples and PCR, CDC, and Gram stain for the subsequent 50 samples. All included patients provided informed consent. The study received prospective ethical approval from the Northwest NHS Research Ethics Committee and was conducted according to the ethical standards set out in the 1964 Declaration of Helsinki, as revised in 2000.

Conventional Diagnostic Culture

Gram stain was carried out immediately on arrival in the laboratory according to the British Society for Antimicrobial Chemotherapy (BSAC) guidelines.[21] The BHI bottles were vortexed for 5 to 10 seconds and 10 µl inoculated onto blood, chocolate, and Sabouraud's dextrose agar plates. Agar plates and a 24-hour subculture of the BHI broth were examined for evidence of bacterial growth after 24 hours and 48 hours of incubation. Sabouraud's dextrose agar plates were examined daily for 14 days for any growth. All isolates were identified using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Bruker, Bremen, Germany).

16S rRNA Gene PCR

Four-hundred µl MagNA pure bacterial lysis buffer (BLB; Roche Diagnostics Ltd., West Sussex, UK) was added to the tubes containing corneal scrapes. The tubes were vortexed for 1 minute and then sonicated and centrifuged at 13,000 rpm for 3 minutes. Four-hundred µl of BLB was transferred to a new tube, heated at 95°C for 10 minutes, and DNA was extracted using the automated Roche Compact system (Roche Diagnostics Ltd.).

PCR mix (20 µl volumes) was prepared using the 16S DNA free master mix kit (Molzym, Bremen, Germany). Forward (AGAGTTTGATCMTGGCTCAG) and reverse (GGACTACCAGGGTATCTAATCCTGTT) primers, that amplify the first 5 variable regions of the 16S rRNA gene, were added to the 2.5 times master mix, containing MolTaq 16S and nuclease-free water, to give a final concentration of 300 nM. PCR conditions were 95°C for 60 seconds, followed by 35 cycles at 95°C for 10 seconds, 54°C for 10 seconds, and 72°C for 50 seconds with a final extension of 72°C for 5 minutes.[22,23] Gel electrophoresis was performed using a 2% agarose gel containing ethidium bromide in 1 times tris-borate-ethylenediaminetetraacetic (TBE) buffer for 25 minutes at 140 v. Any PCR amplicons were visualized under UV light and then purified using an enzymatic method (ExoSap-IT; Affymetrix, High Wycombe, UK) according to the manufacturer's instructions. Cycle sequencing was performed by forward and reverse priming using the Big Dye version 1.1 Terminator Reaction kit (Life Technologies, Paisley, UK). Cycling conditions were: 95°C for 30 seconds, followed by 25 cycles at 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. Sequencing was performed on a ABI3130 genetic analyzer (Life Technologies) and sequence data analyzed using SeqScape software. Consensus sequences of both DNA strands were analyzed using the National Center for Biotechnology Information (NCBI) Basic local alignment search tool (BLAST) software available at http://blast.ncbi.nlm.nih.gov/Blast.cgi. Sequences from corneal scrapes were compared with those available in the GenBank database. Positive identifications of bacteria to genus or species level were made according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.[24] The 16S PCR has been optimized

Table 1. Positive and Negative Corneal Scrapes by Conventional Diagnostic Culture and 16S rRNA Gene Polymerase Chain Reaction

	CDC +ve	CDC -ve	Total
16S PCR +ve	13 (56.5%)	13 (16.9%)	**26**
16S PCR -ve	10 (43.5%)	64 (83.1%)	**74**
Total	**23**	**77**	**100**

+ve = positive; –ve = negative; CDC = conventional diagnostic; PCR = polymerase chain reaction methods.

to not amplify any bacterial DNA, which may be inherent in reagents.[22,23]

Identification of Contaminant Species

Isolates were identified as positive or likely contaminant by the following definitions:

- CDC Positive: Clear organism/s isolated from direct culture or significant organism isolated from enrichment broth culture.
- CDC Contaminant: Present in enrichment broth only and organism consistent with skin flora and a not clearly significant organism (e.g., Coagulase negative Staphylococci [CNS], Diphtheroid, Alpha-hemolytic Streptococci, *Bacillus sp.* non-cereus).
- 16S rRNA gene PCR Positive: ≥ 1+ band positive and organism identified by sequencing, or ≥ 1+ electrophoresis gel band positive and positive on repeat PCR.
- 16S rRNA gene PCR Contaminant: 1+ electrophoresis gel band positive only and negative on repeat PCR testing.

Statistical Analysis

All data collected in the study were entered into an electronic database via Microsoft Excel 2016 and analyzed using SPSS (version 22). The χ^2 was used as indicated for the analysis of categorical variables.

Results

One hundred consecutive patients with clinically suspected bacterial keratitis were included. The mean age of the patients was 49 years (SD = 16.9). The overall isolation rate was 36.0%. 72.2% (26/36) of isolates were detected by 16S rRNA gene PCR and 63.9% (23/36) samples were detected by CDC (P = 0.622; see Tables 1–3). We demonstrated that using a combination of both PCR and CDC detection methods significantly increased the overall isolation rate from 23.0% to 36.0% compared to using CDC alone ($P = 0.04$).

Using the contamination definitions outlined in the methods section, the overall rate of detection of contaminant species for both PCR and CDC was 30.6% (11/36). 19.2% (5/26) of isolates identified by 16S rRNA gene PCR were deemed to be contaminant species compared to 30.4% (7/23) of isolates identified by CDC (P = 0.363). Possible contaminated samples are demonstrated in Table 3.

The CDC and 16S rRNA gene PCR isolate results for presumed pathogenic bacteria are presented in Table 2. In 9 of 83 (10.8%) CDC negative samples, presumed pathogenic bacteria were detected by 16S PCR; *Moraxella nonliquefaciens* was isolated in 2 samples, *P. aeruginosa* in 2 samples, *Acinetobacter sp.* in 1 sample and *Methylobacterium sp.* in 1 sample. In two samples, mixed sequences were found and in one sample the sequence was inconclusive. In 4 out of 78 patients (5.1%) who were 16S PCR negative, presumed pathogenic bacteria was detected by CDC; *Moraxella nonliquefaciens* was isolated in 1 sample, *S. aureus* isolated from the enrichment only in 2 samples, and 1 patient had CNS isolated from the agar plate.

Of the 17 presumed pathogenic isolates detected by CDC, 14 of 17 (82.4%) were identified to the species level and 3 (17.6%) to the genus level. Of the 22 presumed pathogenic isolates detected by 16S rRNA gene PCR, 11 of 22 (50.0%) were identified to the species level, 3 (13.6%) to the genus level, and 9 (40.1%) had inconclusive or mixed sequence results. Of the 13 samples that were positive by both 16S PCR and CDC, there was genus concordance in 7 (53.8%); 5 samples with *Moraxella sp.* and 2 samples with *Pseudomonas sp.* Species concordance was demonstrated in 3 of 13 (23.1%) samples (*Moraxella lacunata*, *Moraxella catarrhalis*, and *P. aeruginosa*). The 3 patients that were found to have polymicrobial infection on CDC were all found to have mixed nonidentifiable sequences on 16S PCR.

Order of Sample Collection

The order of sample collection did not have any effect on the rate of positive detection of microorganisms by PCR; of the 22 PCR positive samples, 11 of 50 were collected as the first scrape and 11 of 50 as the third, respectively ($P = 1.0$).

Table 2. Samples Containing Pathogenic Microorganisms Identified by Conventional Diagnostic Culture and 16S rRNA Gene Polymerase Chain Reaction

Sample	CDC	16S PCR
1	*Moraxella sp.*	*Moraxella sp.*
2	*Moraxella sp.*	*Moraxella nonliquefaciens*
3	*Moraxella lacunata*	*Moraxella lacunata*
4	*Moraxella catarrhalis*	*Moraxella nonliquefaciens*
5	*Moraxella catarrhalis*	*Moraxella catarrhalis*
6	*Moraxella nonliquefaciens*	Nil
7	Nil	*Moraxella nonliquefaciens*
8	Nil	*Moraxella nonliquefaciens*
9	*P. aeruginosa, α hemolytic Strep*	Mixed sequence
10	*P. aeruginosa*	*P. aeruginosa*
11	*Pseudomonas sp.*	*P. aeruginosa*
12	*P. aeruginosa, Aspergillus flavus*	Mixed sequence
13	Nil	*P. aeruginosa*
14	Nil	*P. aeruginosa*
15	*S. aureus* (enrichment only)	Mixed sequence
16	*S. aureus* (enrichment only)	Nil
17	*S. aureus* (enrichment only)	Nil
18	*Enterobacter cloacae, α-hemolytic Strep*	Mixed sequence
19	*Serratia marcescens*	bacterial DNA
20	Nil	*Acinetobacter sp.*
21	Nil	*Methylobacterium sp.*
22	CNS +/− (plate)	Nil
23	Nil	Mixed sequence with *Strep sp.*
24	Nil	Mixed sequence
25	Nil	Bacterial DNA

CDC = conventional diagnostic culture; CNS = coagulase negative staphylococci; PCR = polymerase chain reaction.

Prior Antimicrobial Use

Thirty-four of 100 (34%) of the study samples had received topical antimicrobial treatment prior to sample collection: 21 chloramphenicol, 8 ciprofloxacin, 1 fucithalmic acid, 3 ciprofloxacin and teicoplanin, and 1 patient ciprofloxacin and fucithalmic acid. There was no significant difference in the isolation rate detected either by CDC or PCR between those patients who had or had not received antimicrobial treatment prior to sample collection, 7 of 34 (20.6%) and 19 of 66 (28.2%), respectively ($P = 0.376$). Of the nine patients who had presumed pathogenic bacteria detected by PCR only, two of nine (22%) had received antimicrobial treatment prior to sample collection. In both cases, the antimicrobial used was chloramphenicol and the organism identified by PCR was *P. aeruginosa*. Of the four patients who had presumed pathogenic bacteria detected by CDC only, two of four (50%) had antimicrobial treatment prior to sample collection. In both cases, ciprofloxacin was used and the organism isolated was *S. aureus* (from enrichment only). Of note, these two samples were the only positive result from either PCR or CDC among the patients who received prior treatment with ciprofloxacin.

Discussion

In this study, we demonstrated that a combination of both CDC and 16S rRNA gene PCR significantly increased the overall isolation rate than CDC alone. Although, in CDC negative samples, 16S PCR yielded more results suggestive of potential pathogens (*M. nonliquefaciens* and *P. aeruginosa*) than CDC in 16S PCR negative samples, comparatively more 16S PCR positive samples were found to have inconclusive results compared to CDC. We demonstrated that problems of distinguishing infection from contamination exist with both techniques. Neither order of sample collection nor prior antimicrobial use was demonstrated to affect the isolation rate with either method.

Table 3. Samples Containing Contaminating Microorganisms Identified by Conventional Diagnostic Culture and 16S rRNA Gene Polymerase Chain Reaction

Sample	PCR Sample Collected	CDC Contaminant	PCR Band	PCR Contaminant	Repeat PCR Band
1	Third scrape	CNS (enrichment only)	NEG		
2	Third scrape	CNS (enrichment only)	NEG		
3	Third scrape	CNS (enrichment only)	NEG		
4	Third scrape	Mixed CNS (enrichment only)	NEG		
5	Third scrape	Mixed CNS (from enrichment)	NEG		
6	First scrape	CNS (enrichment only)	NEG		
7	Third scrape	*Bacillus* sp.	+	*Corynebacterium mastididis*	NEG
8	Third scrape	No growth	+	*Propionibacterium acnes*	NEG
9	Third scrape	No growth	+	*Acinetobacter sp.*	NEG
10	First scrape	No growth	+	Bacterial DNA	NEG
11	First scrape	No growth	+	Bacterial DNA	NEG

CDC = conventional diagnostic culture; CNS = Coagulase negative staphylococci; PCR = polymerase chain reaction; NEG = negative; POS = positive.

As far as we are aware, this is the first prospective study to control for sampling methodology and order and also identify possible contaminating organisms in a large patient cohort. The absence of detecting a difference between CDC and PCR may reflect that the study was under powered given the actual CDC and PCR rates, which were lower than expected, that is, a post hoc power calculation is 14% as opposed to the intended 20%. Despite these limitations, the results are comparable to those reported from noncontrolled studies by Kim et al.[18] and Panda et al.[19]. Kim et al.[18] compared CDC from corneal scrapings to 16S PCR from corneal swabs and demonstrated similar CDC and 16S positive isolation rates to our study. Panda et al.[19] compared CDC from samples obtained from the cornea via a cotton tippled applicator to 16S PCR from corneal scrapings in 122 bacterial keratitis patients, reporting 43.5% CDC positive and 45.9% 16S PCR positive. These rates are higher than the isolation rates demonstrated within this study and are likely related to the increased severity of infection seen on presentation compared to a typical UK population.[25,26] Comparatively much smaller studies carried out by Knox et al.,[13] Ameen et al.,[14] Eleinen et al.,[15] Itahashi et al.,[16] and Rudolph et al.[17] demonstrated positive isolation rates for CDC varying between 25% and 57.5% and for PCR 45.5% and 100%.

In infections other than ophthalmic infections, 16S PCR and CDC have a concordance of approximately 90.6%.[27,28] High concordance rates of 70% to 100% are also seen in endophthalmitis.[29,30] This is in contrast to bacterial keratitis, where concordance rates are typically much lower, as demonstrated in our study.[12,18] Lower concordance rates in bacterial keratitis may be attributable to the detection of either colonizing or contaminating microorganisms by CDC/PCR. This is a difficult factor to assess with any certainty. For example, in our study, the one case in which *Moraxella nonliquefaciens* was isolated using CDC but not with PCR and the two cases in which *S. aureus* (an accepted pathogen) were isolated only in enrichment culture but not with PCR may represent failure by PCR or contamination in CDC.

PCR is capable of 10^6 to 10^7-fold amplification of a single copy of template DNA, making minor contamination of the PCR mixture with exogenous DNA a problem.[31] This is highlighted by Kim et al.[18] who demonstrated high 16S PCR false positive rates for apparently nonpathogenic organisms with control "air" swabs and by Corless et al.[32] who demonstrated amplification of 16S rRNA sequences when no exogeneous DNA had been added to a negative control. In our study, in the cases where CDC was negative but *Methylobacterium* or unidentifiable sequences of bacterium DNA were identified by PCR, this could represent a similar contamination process either with environmental or commensal ocular surface bacteria or bacteria introduced during sample processing. PCR reagent treatment with UV irradiation, 8-methoxypsoralen activity facilitated by UV, and DNase alone and in combination with restriction digestion have all been demonstrated to reduce PCR sensitivity and could improve specificity and interpretation of PCR results.[32]

Lower concordance rates between CDC and PCR in bacterial keratitis could also be due to processing

biases. CDC results are likely to be biased to more fast-growing microorganisms, which can be easily cultivated on a standard media. In our study, the PCR negative but CDC positive results may have resulted from difficulties in breaking the cell walls of Gram-positive organisms during the DNA extraction process. Using a more effective method, such chemical lysis with proteinase K or mechanical disruption, may result in more reliable PCR detection.

Polymicrobial keratitis has an 2% to 8% incidence rate in the literature and is associated with larger corneal infiltrate size and greater mean duration for resolution of infection.[31,32] In our study, all three samples with mixed growth on CDC had an unidentified mixed sequence on PCR. 16S rRNA gene sequencing using bulk PCR products cannot be applied to polymicrobial bacterial specimens as the presence of multiple templates results in superimposed reads that are mostly uninterpretable.[35]

Prior antimicrobial use had no effect on the positivity of samples from 16s rRNA gene PCR in our study. This was unexpected because PCR can amplify dead bacteria, which are unable to grow for bacterial culture, and is in contrast to several studies who found that antibiotics before specimen collection was a significant factor in reducing culture positivity.[12,19] The comparatively small numbers of patients who had used topical antimicrobial therapy in our study may account for the fact that we saw this lack of effect.

For the purposes of this study, only cases of suspected bacterial keratitis cases were investigated using CDC and PCR. BHI bottles were, however, inoculated onto Sabouraud's dextrose agar plates for fundal growth. Internal transcribed spacer (ITS), 28S rRNA, and 18S rRNA PCR have all been demonstrated to increase fungal detection rate in culture-negative cases and fungal PCR would be a useful adjunct to culture.[36–38] Multiplex real-time PCR assays are also being increasingly used for the diagnosis of viral keratitis.[39] Until newer techniques, such as shotgun metagenomics, become more clinically interpretable and cost effective, a PCR assay that combines broad range primers for bacterial, fungal, and viral detection is an interesting avenue that would allow rapid detection in keratitis cases where the diagnosis is not clear or when the keratitis is polymicrobial.

In conclusion, although we did not find 16S rRNA gene PCR to have a significantly higher sensitivity for detecting the presence of bacteria in corneal scrape samples compared to CDC, additional presumed pathogenic bacteria were identified in CDC negative samples. PCR offers advantages over culture of rapid analysis and the ability to detect organisms from very low starting material, however, contamination and the fact that PCR does not provide antimicrobial susceptibility data means that it should still be continued to be used as an adjunct technique to CDC in diagnosing bacterial keratitis.

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