# Identification of unknown ocular pathogens in clinically suspected eye infections using ribosomal RNA gene sequence analysis

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## Abstract

DNA sequence-based identification of pathogens from ocular samples of patients with clinically suspected eye infections was accomplished using 16S and internal transcribed spacer (ITS) ribosomal RNA gene sequence analysis. PCR was positive for 24 of 99 samples tested. Both culture and 16S rDNA sequence analysis identified *Pseudomonas aeruginosa*, streptococci and Enterobacteriaceae. Isolates misidentified as *Burkholderia cepacia* by biochemical tests were identified as *Ralstonia mannitolilytica* by 16S rDNA sequence analysis. Sequence analysis identified the following microorganisms from 19 culture-negative samples: *Haemophilus influenzae*, *Sphingomonas* sp., *Klebsiella pneumoniae*, *Staphylococcus haemolyticus*, *Morganella morganii*, *Mycobacterium* sp., *Chryseobacterium* sp., *Pseudomonas saccharophila* (*Xanthomonas*) and the fungus, *Phaeoacremonium inflatipes*.

**Keywords:** 16S rDNA, DNA sequence analysis, eye infections, internal transcribed spacer rDNA, ocular pathogens

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## Introduction

Identifying the aetiological agent in clinically suspected eye infections can be frustrating. Conventional laboratory tests such as Gram stain and culture may give negative results because of the relatively small sample size of aqueous (100–150 $\mu$L) and vitreous (200–400 $\mu$L) humour. Other possible limitations are the adherence of the microorganism to solid surfaces (intraocular lens and capsule) thereby contributing to a low microbial load in the liquid sample, previous empirical use of broad-spectrum antibiotics and the fastidious nature of some microorganisms [1]. Cognizant of these limitations, 16S rRNA gene sequence analysis [2] and rRNA internal transcribed spacer (ITS) region [3] have been reported as excellent choices for identifying unknown or non-culturable bacteria or fungi, respectively, in the absence of *a priori* knowledge.

This paper describes the PCR-based detection and DNA sequence-based identification of microbial pathogens from ocular samples of patients with clinically suspected eye infections by 16S rRNA (bacteria) and ITS rRNA (fungi) gene sequence analysis. These molecular techniques were compared with conventional microbiological methods.

## Materials and Methods

Ethical clearance for the study was given by the Institutional Ethics Review Board. Ocular samples, consisting of vitreous aspirate, anterior chamber tap, and other specimens from the eye, were obtained with informed consent from patients diagnosed with clinically suspected eye infections at the International Eye Institute of St Luke's Medical Centre and other metropolitan hospitals (Table 1). Each sample was analysed by bacterial culture (MacConkey agar, blood agar and tryptic soy agar), Gram stain and PCR. Biochemical characterization of the isolates using the Becton Dickinson BBL Crystal Identification System was performed (Becton Dickinson, Franklin Lakes, NJ, USA).

DNA was extracted using the QIAGEN QIAamp DNA Mini kit (Qiagen, Valencia, CA, USA). PCR was performed to amplify the 16S rRNA gene using the QIAGEN Taq PCR Core kit, as follows: PCR mix—PCR-grade $H_2O$ to make final volume of 25 $\mu$L; 2.5 $\mu$L 10× buffer; 3.0 $\mu$L Q Solution; 0.2 $\mu$L dNTPs; 0.2 $\mu$L forward primer (8FPL: 5′-AGT TTG ATC CTG GCT CAG-3′); 0.2 $\mu$L reverse primer (806R: 5′-GGA CTA CCA GGG TAT CTA AT-3′; [4]; and 0.18 $\mu$L *Taq* polymerase; 2 $\mu$L (*c.* 10 ng) of genomic DNA extract or PCR-grade water was added. The reaction was placed in a G-Storm Gene Technologies Gradient Thermal Cycler (Gene Technologies, Braintree, Essex, UK) set to the following conditions: initial denaturation at 95°C for 10 min, followed by 30 cycles of: denaturation at 95°C for 1 min, annealing at 51°C for 1 min and extension at 72°C for 2 min, and then,

**TABLE 1. Ocular samples from cases of infectious uveitis used in this study**

| Specimen | Number |
|---|---|
| Anterior chamber tap | 43 |
| Vitreous aspirate | 47 |
| Corneal scraping | 3 |
| Intraocular lens | 1 |
| Eyeball | 1 |
| (–) Controls—Anterior chamber tap | 4 |
| Total | 99 |

final extension at 72°C for 7 min. The 834-base-pair amplified product was visualized by agarose gel electrophoresis.

Samples that were negative for 16S rRNA gene were then analysed for rRNA ITS region by PCR as follows: PCR Mix—PCR-grade $H_2O$ to make final volume of 25 $\mu$L; 2.5 $\mu$L 10× buffer; 3.0 $\mu$L Q Solution; 0.3 $\mu$L dNTPs; 0.2 $\mu$L forward primer (ITS-1: 5′-TCC GTA GGT GAA CCT GCG G-3′); 0.2 $\mu$L reverse primer (ITS-4: 5′-TCC TCC GCT TAT TGA TAT GC-3′) [5]; and 0.18 $\mu$L *Taq* polymerase; 2 $\mu$L (*c.* 10 ng) of genomic DNA extract or PCR-grade water was added. The reaction was placed in a G-Storm Gene Technologies Gradient Thermal Cycler set to the following conditions: initial denaturation at 94°C for 5 min, followed by 40 cycles of: denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 90 seconds and then, final extension at 72°C for 7 min. The 560-base-pair amplified product was visualized by agarose gel electrophoresis.

The PCR products were submitted to Macrogen, Inc. (Seoul, Korea) for DNA sequencing. DNA sequences were analysed using CHROMASLITE (Technelysium Pty. Ltd., Tewantin, QLD, Australia) and BIOEDIT (Ibis Therapeutics, Carlsbad, CA, USA) software. Comparison with 16S and ITS rRNA gene sequences in GenBank (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD) was performed using the BLAST.

## Results

Ninety-nine samples from 62 patients, including four control (non-infected) samples (Table 1), were tested for the presence of microbial pathogens by direct Gram-staining, conventional microbiological culture techniques and PCR. Five samples were shown to harbour Gram-negative bacilli and the rest, including the controls, were negative by direct Gram stain. Seven (7%) of the 99 samples produced colonies when inoculated in MacConkey agar, blood agar, or tryptic soy agar media. All four control samples were negative for the presence of bacteria by direct Gram stain, conventional culture and PCR. All water blank reactions were negative for amplified rDNA.

The PCR of the 16S rRNA gene was positive in 24 samples from patients with clinically suspected eye infections. Of these, 17 were deemed non-culturable in three culture media used. Identification of bacteria was made on the basis of DNA sequences (BLAST) found in GenBank (National Center for Biotechnology Information, National Institutes of Health). Table 2 presents the results of the biochemical tests and 16S rRNA gene sequence analyses in identifying the pathogen bacteria in a sample. The percentage similarity of the 16S rRNA sequences from each sample in comparison to reference sequences in the GenBank database is also given. The isolates from two samples belonging to one patient was misidentified as *Burkholderia cepacia* by biochemical tests, but was unequivocally identified as *Ralstonia mannitolilytica* by 16S rRNA gene sequence analysis. The misidentification of *R. mannitolilytica* as *Pseudomonas fluorescens* or *Burkholderia cepacia* has been reported in nosocomial infections, such as meningitis [6] and cystic fibrosis. The case reported here involves a 63-year-old man who had worked as a gardener in a florist shop who sought medical attention for a 'red eye' condition. *Pseudomonas aeruginosa* was identified by both culture and 16S rRNA gene sequence analysis in two samples (vitreous aspirate and corneal scraping) from a patient (53-2007) who had undergone cataract surgery via phaecoemulsification and developed a painful 'red eye' condition because of inflammation 4 days after surgery. For sample 04a-2004, results gave 99–97.9% identity with an uncultured bacterium, 98.7–98.5% with *Streptococcus oralis* and 98–97% with an uncultured *Streptococcus* sp. Biochemical characterization of the isolate identified it as either *Streptococcus sanguis* or *Streptococcus constellatus*. In the case of sample 05b-2004, both the biochemical tests and 16S rRNA gene sequence signified the presence of bacteria belonging to the Enterobacteriaceae in the sample, but were unable to identify the exact species.

Table 3 gives the results for 17 of 24 samples sequenced that failed to produce colonies on conventional media, but harboured bacterial pathogens that were identified as *Sphingomonas* sp. (12a/b-2004), *Pseudomonas saccharophila* (13b-2004), *Haemophilus influenzae* (22b/c-2005), *Klebsiella pneumoniae* (24-2005 and 44a/b-2006), *Chryseobacterium* sp. (27-2005) *Staphylococcus haemolyticus* 35a/b-2005), *Mycobacterium abscessus* and *Mycobacterium* sp.(38a and b-2005, respectively), *Morganella morganii* (46a-2006, 47a/b-2006) and an uncultured bacterium from corneal scraping (49-2006).

Only one sample (13a-2004) tested positive for fungal rRNA ITS region with DNA sequence analysis showing 99% homology to *Phaeoacremonium inflatipes*.