

Autofluorescence signatures of seven pathogens: preliminary *in vitro* investigations of a potential diagnostic for Acanthamoeba keratitis

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Abstract

Purpose: Acanthamoeba keratitis can cause devastating damage to the human cornea and is often difficult to diagnose by routine clinical methods. In this preliminary study, we investigated whether *Acanthamoeba* may be distinguished from other common corneal pathogens through its autofluorescence response. While only a small number of pathogens were studied, the identification of a unique *Acanthamoeba* signature would indicate that autofluorescence spectroscopy as a diagnostic method merits further investigation.

Methods: Samples of seven common pathogens (*E. coli*, *S. aureus*, *P. aeruginosa*, *E. miricola*, *A. ruhlandii*, *C. albicans* and *A. castellanii*) in solution were excited with ultraviolet light at a number of successive, narrow wavebands between 260 nm and 400 nm, and their fluorescence response recorded. Principal Component Analysis was used to allow better visualisation of the differences in response to UV light for different species.

Results: *Acanthamoeba* was found to possess a characteristic autofluorescence response and was easily distinguished from *E. coli*, *S. aureus*, *P. aeruginosa*, *E. miricola*, *A. ruhlandii* and *C. albicans* over a wide range of excitation wavelengths. We also found a clear discrimination between *E. coli*, *C. albicans* and *P. aeruginosa* at an excitation wavelength of 274 nm, while *E. miricola*, *S. aureus* and *A. ruhlandii* could be separated using an excitation wavelength of 308 nm.

Conclusions: Our results, while preliminary, indicate that autofluorescence spectroscopy shows promise as a diagnostic technique for keratitis. We intend to

expand the set of pathogens studied before assessing the feasibility of the technique *in vivo* by introducing cultures onto pig corneas.

Introduction

Microorganisms exposed to ultraviolet light exhibit an intrinsic fluorescence, or autofluorescence, due to the presence of the fluorescent amino acids tryptophan, tyrosine and phenylalanine and co-enzymes such as NADH. The fluorescence emission from tryptophan in particular is strongly influenced by its local environment, for example the structure of the protein in which it exists and any association of that protein with substrates or other macromolecules¹. Hence, two species of microorganism illuminated with the same ultraviolet light may have very different autofluorescent responses.

The phenomenon of autofluorescence has previously been exploited to identify bacteria relevant to both the food industry and the medical sciences²⁻⁸. It has also been shown that it is possible to distinguish between bacteria and fungi using their autofluorescence signatures⁹. Recently, autofluorescence spectroscopy has been used to directly identify 37 common bacterial and fungal pathogens in blood cultures, with potential application to the clinical management of sepsis. An algorithm was developed that could correctly classify 99.6% of the unknown

samples to the Gram staining level, 99.3% to the family level, and 96.5% to the species level using autofluorescence measurements alone¹⁰. Similarly, the urine of patients with urinary tract infections has been shown to exhibit an increase in autofluorescence¹¹. Aside from microbiology, there are a number of additional potential clinical applications of autofluorescence spectroscopy, including the monitoring of tissue damage¹² and the diagnosis and monitoring of various cancers (see, for example, the review by Liu et al. 2013¹³), and autofluorescence endoscopies are already routinely used to identify potential biopsy sites. While autofluorescence spectroscopy has thus already shown promise as a method of identifying different bacteria, fungi and tissue types, autofluorescence measurements for the identification of *Acanthamoeba* have not previously been investigated.

Microbial keratitis affects up to 710 per 100,000 population per year¹⁴.

Acanthamoeba is a ubiquitous, free-living protozoan that can cause devastating ulceration of the human cornea, particularly in soft contact lens wearers, which can lead to blindness and even loss of the eye in 2% of patients¹⁵. The increasing threat of *Acanthamoeba* keratitis has been well reported¹⁶⁻¹⁸ and it is clear that prompt diagnosis and appropriate targeted therapy are essential to a good prognosis^{14,15}. Late diagnosis leads to the increased need for surgical intervention and worse visual outcomes¹⁹. However, identifying *Acanthamoeba* as the cause of a corneal infection by routine clinical methods is difficult and often leads to delays in diagnosis. Microbiological culture remains the 'gold standard' but these tests are only positive in 52.5% - 67% of cases²⁰. Often a

combination of culture, confocal microscopy, histological examination of biopsy specimens and detection of *Acanthamoeba* DNA by polymerase chain reaction is used to arrive at the diagnosis¹⁵.

Here, we have demonstrated that *Acanthamoeba* can be distinguished *in vitro* from other common corneal pathogens by its autofluorescence signature.

Materials and Methods

The autofluorescence signatures of five bacteria, one yeast and one strain of *Acanthamoeba castellanii* (ATCC 50370) were studied. Cultures of *Escherichia coli* (ATCC 8739), *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 9027), *Elizabethkingia miricola* (3AS) and *Achromobacter ruhlandii* (S4) were grown on trypticase soy agar at 32° C for 24 hours in air. *Candida albicans* (ATCC 10231) was grown on Sabouraud Dextrose Agar at 32° C for 24 hours in air. *A. castellanii* trophozoites were cultured in a semi-defined axenic broth medium²¹. All samples, except *Acanthamoeba*, were then suspended in Dulbecco's phosphate buffered saline (DPBS) for the autofluorescence measurements. The optical density at 600 nm (OD₆₀₀) of the DPBS suspended bacterial and yeast samples were ~0.1.

For the *A. castellanii*, the trophozoites were washed ×3 in 1/4 strength Ringer's solution or deionized water by centrifugation at 500 ×g for 5 minutes. Each *A.*

castellanii sample contained $\sim 1 \times 10^5$ trophozoites. It was important that the trophozoites were washed thoroughly since the axenic broth medium in which they were cultured was found to be highly fluorescent. The use of two different solutions for the *Acanthamoeba* samples was intended to show that any differences between the autofluorescence of the amoeba and that of the other species were consistent and not environment dependent.

Excitation wavelength-resolved autofluorescence spectroscopy was conducted using an Ocean Optics HPX-2000 Xenon arc lamp as the excitation source. The lamp was connected via an optical fibre to a Newport Cornerstone 130 motorised monochromator, which allowed a small (~ 1 nm) region of light to be isolated within the lamp's 185 – 2000 nm output. The selected light was directed onto the surface of each sample using an Ocean Optics QR200-7 UV reflectance probe. The probe consisted of a ring of six illumination fibres and a central fibre branching away from the others to direct reflected light and autofluorescence to an Ocean Optics Maya spectrometer (sensitive in the range 250 – 700 nm). Each sample was illuminated at 71 excitation wavelengths, covering the range 260 – 400 nm in 2 nm steps. Three consecutive scans of a sample were performed before the probe was moved to illuminate the next species. This process was repeated five times so that a total of 15 spectra at each excitation wavelength were collected for each species. Scans of all pathogens except *Acanthamoeba* were performed over two consecutive days –three sets of scans on day one and two on day two. Fresh samples were produced each day from the cultured plates. All *Acanthamoeba* scans were performed in a single day. The exposure time per

illumination wavelength was 0.5 seconds. A schematic of the equipment is given in Figure 1.

The collected 71 spectra from each scan were normalised to the maximum intensity seen at an excitation wavelength of 290 nm during that scan. This had the effect of removing any differences in concentration between samples, which result in different fluorescence intensities but do not affect the shape of the fluorescence response. It is important that the identification of a species by its autofluorescence is not concentration dependent since we will clearly have no control over the concentration of pathogens *in vivo*.

Results

Excitation Emission Matrices (EEMs) were produced for each sample by plotting each set of 71 spectra as excitation versus emission response, as shown in Figure 2. The EEMs shown are normalised to the maximum of the fluorescence response at an excitation wavelength of 290 nm. The *Acanthamoeba* autofluorescence is clearly different to that of the other microorganisms, exhibiting a “comma shaped” morphology. While the shape of the fluorescence signature was similar for all other samples (with the exception of *P. aeruginosa*, which exhibits a secondary fluorescence due to the presence of the fluorescent pigment pyoverdine), small differences can be seen. The fluorescence signal is concentrated in the excitation-emission region associated with amino acid fluorescence. We attribute most of this signal to tryptophan, since phenylalanine

fluorescence is significantly weaker and tyrosine fluorescence in proteins is generally quenched by a number of factors including nearby carboxyl groups and amino groups¹.

We applied Principal Component Analysis (PCA) to the emission spectra obtained at a number of excitation wavelengths which showed the clearest variation between species. The data were transformed onto a set of uncorrelated variables called Principal Components, such that the first Principal Component accounts for as much of the variation between spectra as possible, and each successive Principal Component accounts for as much variation as possible while also being uncorrelated with all previous Principal Components. The differences between spectra from different species may then be more easily visualised by creating 2D scatter plots using Principal Components as axes.

We found that *Acanthamoeba* and *P. aeruginosa* could be readily distinguished from the other samples over a wide range of excitation wavelengths, as expected from the appearance of their EEMs. While we were unable to identify a single wavelength at which all of the species studied could be unambiguously identified, we were able to distinguish between all samples using a combination of two excitation wavelengths. For example, *Acanthamoeba*, *P. aeruginosa*, *E. coli* and *C. albicans* were all well separated in PCA plots for an excitation wavelength of 274 nm, but the remaining three species overlapped. By applying a second PCA to spectra obtained at an excitation wavelength of 308 nm we were able to separate these three species. The emission spectra obtained for excitation wavelengths of 274 nm and 308 nm for each species are shown below the

relevant EEM in Figure 2. PCA plots for 274 nm and 308 nm excitation are shown in Figure 3.

Discussion

These data demonstrate that *Acanthamoeba* can be distinguished from a subset of other common corneal pathogens based on its autofluorescence characteristics. Furthermore, the excitation-emission matrices, resolved to the nanometre level for excitation wavelength, provide characteristic spectral fingerprints for all the corneal pathogens tested. Discrimination of *Acanthamoeba* is unambiguous at a number of excitation wavelengths, including 274 nm, as shown in Figure 3. We were also able to identify a combination of two excitation wavelengths which allowed the discrimination of all species studied.

The increasing incidence of *Acanthamoeba* worldwide and its significant morbidity for a younger population makes it an economically important healthcare problem¹⁶⁻¹⁸. The limitations of current diagnostic techniques and the resulting delay in diagnosis lead to unnecessary suffering and loss of vision. Microbiological cultures may take weeks to become positive and sensitivities are often low, in the range 0 to 68%^{20,22}. PCR is reported to offer marginally better sensitivity for infections in the superficial layers of the cornea but is more expensive, requires technical expertise, is dependent on the sampling methods and is not a widely available technique²³. *In vivo* confocal microscopy is rapidly becoming the preferred technique to early diagnosis at the time of patient

presentation to the eye emergency department. However, while some studies demonstrate sensitivities and specificities up to 94 and 93%, respectively^{24,25}, if the observers are not highly trained then these parameters rapidly fall to 56 and 84%²⁶. Therefore, there remains a clear need for a rapid and accurate diagnostic test for *Acanthamoeba* keratitis.

Producing the high resolution spectral fingerprints for individual corneal pathogens takes minutes at low energy levels of illumination (typically 0.4 millijoule for a complete scan assuming one exposure per excitation wavelength). Such timescales would be acceptable for clinical examination of the corneal ulcers and the spatial resolution of the system is appropriate to the size of a corneal ulcer. The use of principal component analysis will allow the authors to select a subset of exciting wavelengths for sensitive and specific *Acanthamoeba* identification and further reduce the time for each clinical examination.

However, the ability of autofluorescence spectral fingerprints to differentiate *Acanthamoeba* from other corneal pathogens is not necessarily reproducible *in vivo* for several reasons. Firstly, *Acanthamoeba* exists in different stages of a lifecycle which involves an actively replicating and virulent trophozoite and an inactive and highly resistant cystic form²⁷. The spectral fingerprint during an infection may depend on where the majority of *Acanthamoeba* organisms are within this life cycle. The organisms examined in this study were in the

trophozoite phase due to the way the cultures were prepared. However, it is not possible to accurately replicate *in vivo* conditions of an active infection in the laboratory. Secondly, *in vivo* autofluorescence signals will be affected by structural components of the cornea and also the cellular immune response to infection. It may be possible to reduce the background fluorescence by scanning a corneal scrape inoculated into a spectroscopic medium rather than directly observing the infected eye. The feasibility of this technique may be investigated by using titration experiments to determine the lower limit of detection in liquid suspension. Thirdly, while repeat measurements of the samples studied in this initial investigation were performed, only one culture of each pathogen was used: further cultures should be measured to show that the results are fully reproducible. Finally, the range of potential corneal pathogens that need to be distinguished from *Acanthamoeba* is wider than those used in this study. In order to assess the feasibility of this as an *in vivo* technique, we intend to introduce cultures of *Acanthamoeba* and a wider range of other microorganisms which commonly cause keratitis onto pig corneas and assess the autofluorescence spectral fingerprints.

In summary, *Acanthamoeba* appears to possess a distinctive autofluorescence signature that allows it to be easily differentiated from other microorganisms using fluorescence spectroscopy. This feature may be exploited to develop a method for the rapid diagnosis of *Acanthamoeba* keratitis.

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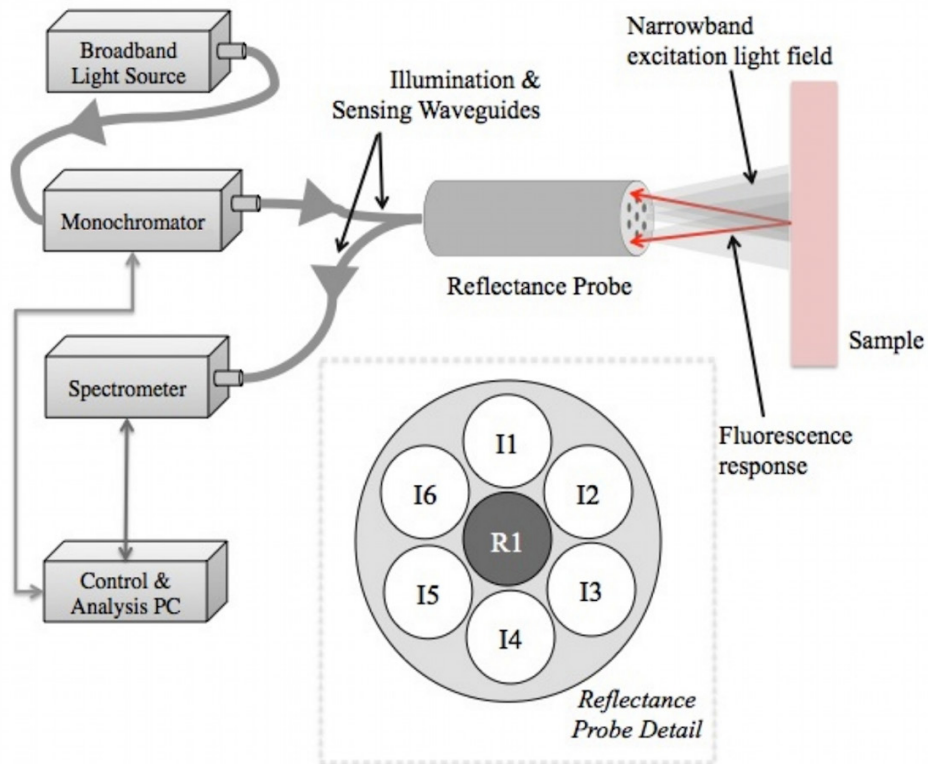
<http://www.cdc.gov/parasites/acanthamoeba/biology.html> (last accessed: 9

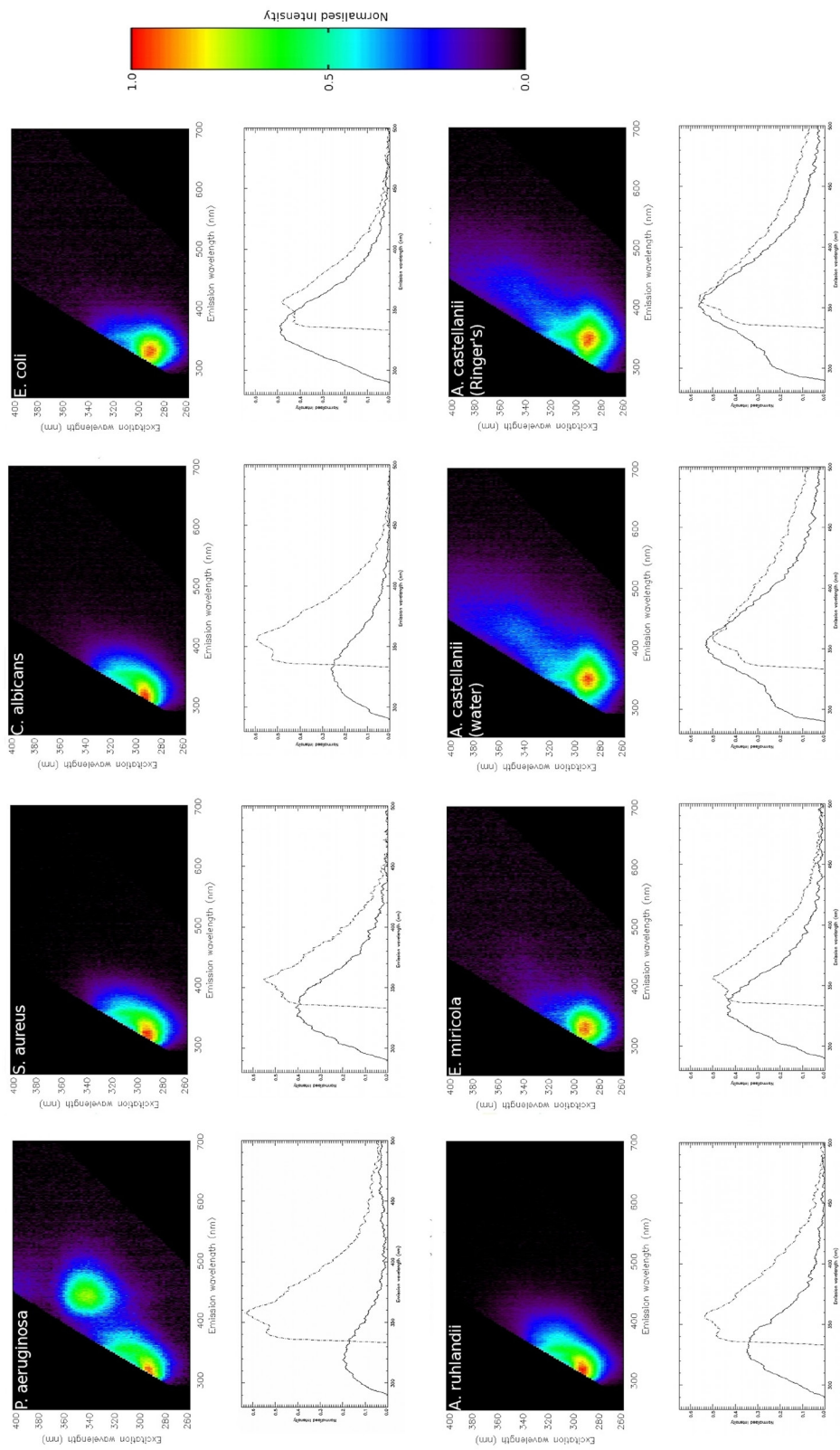
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FIGURE 1. Schematic of autofluorescence spectroscopy equipment. The reflectance probe inset shows the six illuminator (I) fibres and single read (R) fibre.

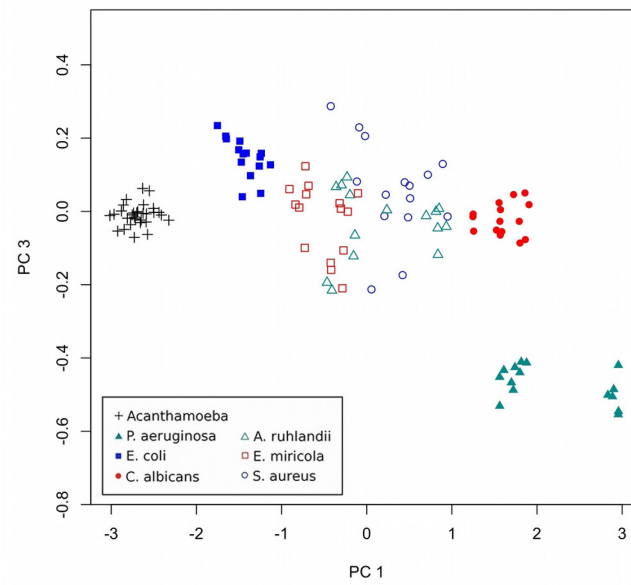
FIGURE 2. Example Excitation-Emission Matrices (EEMs) for the microorganisms studied. Clockwise from top left: *P. aeruginosa*, *S. aureus*, *C. albicans*, *E. coli*, *A. castellanii* in Ringer's solution, *A. castellanii* in deionized water, *E. miricola*, *A. ruhlandii*. The panel below each EEM shows the fluorescence response for the two excitation wavelengths used for Principal Component Analysis: 274 nm (solid line) and 308 nm (dashed line).

FIGURE 3. PCA scatter plots showing the first and third Principal Components for all samples at an excitation wavelength of 274 nm (upper panel) and the first and second Principal Components at 308 nm for the subset of samples that could not be separated at 274 nm (lower panel).





$\lambda_{\text{ex}} = 274 \text{ nm}$



$\lambda_{\text{ex}} = 308 \text{ nm}$

