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Confocal autofluorescence identification of bacteria, fungi, and acanthamoeba in infected porcine cornea models

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ABSTRACT

Current standard in the clinical diagnostic of corneal infection *in vivo* is reflectance confocal microscopy using photons in the wavelength range of 600 s nm. While pathogens with large spatial features (~10 μm) such as acanthamoeba and fungal species can be identified, smaller pathogens such as bacteria (~1 μm) are difficult to detect. Furthermore, reflected light produces images anywhere there are index of refractive index changes. The non-specificity of such images can contribute to confusion in pathogen identification. In this work, we used 488 nm to excite autofluorescence in nine common pathogens (4 bacteria, 3 fungi, and 2 acanthamoeba species) inoculated in *ex vivo* porcine corneas. We found that the detected green autofluroescence is sufficient to clearly image the pathogens. Histograms of the autofluorescence intensity can also act as a metric in pathogen classification.

1. Introduction

Corneal infection (infectious keratitis) is a global health problem. In 2010, it is estimated that 930,000 regular clinical visits and 58,000 emergency room visits occurred for health issues related to keratitis or contact lens use. Of these visits, 76.5% of the patients were prescribed with antimicrobial agents. Overall, 175 million dollars in direct health care were incurred. A more recent study shows that 113–799 per 100 000 of the population in South Asia are affected by corneal ulcer [1,2]. In suspected corneal infection, conventional method of pathogen identification is microbiology in nature in which scrape from patients are cultured and imaged. This is a time-consuming process and may delay in proper treatment [3]. A fast clinical imaging technique is *in vivo* reflectance confocal microscopy. Based on the reflection from interfaces of mismatched indices of refraction, this imaging modality demonstrated to be able to image a number of corneal conditions such as cross-linking, neuropathy, and pathogen identification [4–8]. More recently, multiphoton fluorescence and second harmonic generation (SHG) microscopy has also demonstrated efficacy in imaging corneal pathologies including keratoconus and infectious agents including bacteria, fungi, and acanthamoeba [9–13]. However, the high laser power needed to generate the multiphoton effect has limited the use for ophthalmologic imaging diagnostics.

In vivo reflectance confocal microscopy is effective in identifying pathogens with large spatial features (~ 10 μm) such as acanthamoeba and fungal species. Smaller pathogens such as bacteria are difficult to detect. In addition, confocal reflectance microscopy suffers from the problem of non-specificity in that a signal is recorded anywhere there are index of refractive

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index changes within the specimen. This effect may contribute to uncertainty in pathogen identification. Therefore, if one-photon excited autofluroescence is sufficient for pathogen imaging, the increased specificity may add to enhanced signal contrast, thus improving clinical diagnostics of infectious keratitis.

In this work, we test the efficacy of the use of 488 nm in exciting autofluorescence from nine pathogens (4 bacteria, 3 fungi, and 2 acanthamoeba species) commonly found in corneal infections. To simulate infection, these pathogens were inoculated in *ex vivo* porcine cornea models and imaged through confocal autofluroescence microscopy. Autofluroescence in the green part of the visible spectrum (500–550 nm) was used for imaging.

2. Materials and methods

2.1. Pathogen culture

Pathogen culture in porcine cornea models.

In all, we cultured and inoculated *ex vivo* porcine corneas with 4 bacterial strains (*Staphylococcus aureus* (BCRC 12154, Bioresource Collection and Research Center, Hsinchu, Taiwan), *Pseudomonas aeruginosa* (BCRC 11633), *Streptococcus pneumoniae* (BCRC 14733), and *Neisseria gonorrhoeae* (ATCC® 19424™, American Type Culture Collection (ATCC), Manassas, VA 20110 USA), 3 fungal species (*Candida albicans* (BCRC 21538), *Aspergillus flavus* (BCRC 30165), and *Fusarium solani* (BCRC 34564)), and 2 acanthamoeba species (*Acanthamoeba castellanii* (ATCC® 50370™), and *Acanthamoeba polyphaga* (ATCC® 30461™)). These pathogens are commonly found during the clinical diagnostics of infectious keratitis patients. Culturing conditions for the 9 pathogens can be different. *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Streptococcus pneumoniae* were cultured in Tryptic Soy Broth at 37 °C. *Neisseria gonorrhoeae* was cultured in ATCC® Medium 814 at 37 °C and 5% CO_2. The fungal and acanthamoeba species were cultured at 25 °C. *Candida albicans* was cultured in YM Broth, *Aspergillus flavus* in CZAPEK'S Broth, and *Fusarium solani* in Potato Dextrose Broth. Both acanthamoeba specines were grown in ATCC® Medium 712.

To obtain the cornea specimens, porcine eyeballs obtained from local markets at 9 AM. Corneas were then extracted using 8 mm trephine. The excised corneas were then sterilized with 200 U/ml of Penicillin Streptomycin (Gibco™) in 0.01 M PBS buffer (Sigma-Aldrich, St. Louis, MO) and rinsed with PBS buffer. To simulate the infection process, 26G needles were used to cut three parallel wound approximately 1 mm deep on the corneal surface. Next, 10 μl of solution containing the pathogens are dropped onto the artificial wounds. The cornea specimens were then allowed to continue for 5 days prior to fixation in formalin for 24 h. Cornea specimens prepared in this manner were then imaged through confocal autofluorescence microscopy.

2.2. Confocal microscope setting

The confocal autofluroescence microscopy experiment was performed on a commercial system (LSM 510 Meta, Zeiss, Germany). The excitation light was the 488 nm line and the power used was 0.2 mW. The objective used was an oil immersion lens (Fluar 40× /NA 1.3, Zeiss). Autofluorescence was collected in the epi-illuminated geometry by the objective. Autofluorescence between 500 and 550 nm was collected for imaging. Image analysis was performed with ImageJ.

3. Results and discussion

After inoculation of bacteria onto the porcine cornea specimens, the cornea specimens were imaged. Shown in Fig. 1 are representative autofluroescence images of 4 types of bacteria used in this study (Staphylococcus aureus, Pseudomonas aeruginosa, Streptococcus pneumoniae, and Neisseria gonorrhoeae). As shown in the images, all 4 bacterial species are autofluroescent and often aggregate to form dense colonies within the corneas.

In comparison, representative confocal autofluorescence images of fungal species inoculated in porcine corneas are shown in Fig. 2. Of the 3 fungal species, Aspergillus flavus and Fusarium solani belong to mold and can be identified by the presence of autofluorescent spores (white arrows) and filamentous hyphae (yellow arrows). Morphological, hyphae of Aspergillus flavus is considerably thinner in thickness compared to that of Fusarium solani. On the other hand, *Candida albicans* is a yeast. The individual yeast cells are also autofluroescent and can be clearly distinguished from mold by the absence of hyphae. The third class of corneal pathogens we imaged is acanthamoeba. In contrast to bacteria and fungi, acanthamoeba are significantly larger. Fig. 3 shows confocal autofluorescence images of the 2 acanthamoeba species we imaged. In both cases, the acanthamoea cysts are clearly visualized by the strongly autofluorescent cell walls with relatively weak intracellular autofluorescence.

In addition to morphological autofluorescence imaging, we also analyzed the relative autofluooescence intensities of the 9 pathogens. After eliminating signal background, correction for differences in the use of pinhole sizes and detector gains, histograms of the autofluorescences signal can be obtained (Fig. 4). At the same excitation power, the histograms identify the relative autofluroescence strengths of the pathogens.

Histograms in Fig. 4 can be further used to calculate the average autofluorescence intensities. For all 9 pathogens, we calculated the average autofluorescence intensities for 3 images of each pathogen. The standard deviations from the three measurements are also computed (Table 1).