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Assessment of biofilm formation of *E. meningoseptica*,*D. acidovorans*, and *S. maltophilia* in lens cases and their growth on recovery media

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ABSTRACT

Purpose: Bacterial biofilm formation in contact lens cases is a risk factor in the development of both microbial and infiltrative keratitis. This investigation evaluated three emerging pathogens: *Stenotrophomonas maltophilia*, *Elizabethkingia meningoseptica*, and *Delftia acidovorans* for biofilm formation and metabolic activity in lens cases. Also, growth of these bacteria on different media was assessed to optimize recovery conditions.

Methods: The three bacteria were incubated in lens cases with different concentrations of tryptic soy broth. Biofilm formation was evaluated by measuring metabolic activity using MTT and enumerating the number of viable bacteria. To determine the optimal recovery media, dilutions of these microorganisms were plated on six different media. The number of colony forming units (CFU) was recorded after 48, 72, and 96 h of incubation at 32 °C and 37 °C for *S. maltophilia*, and at 37 °C for *E. meningoseptica* and *D. acidovorans*.

Results: All three microorganisms established biofilms in the lens cases, with significant numbers of CFU recovered. Biofilms of *S. maltophilia* and *E. meningoseptica* were metabolically active. Significant reduction in metabolic activity and number of viable *S. maltophilia* occurred when the incubation temperature was raised from 32 °C to 37 °C ($p < 0.05$). The metabolic activity of the biofilms increased with greater organic load present. The highest percent recovery for all three organisms was given by Columbia blood agar, followed by chocolate.

Conclusion: Based on the results, the presence of the three emerging pathogens present in lens cases and from corneal isolates can be accurately determined if proper growth media and incubation temperatures are utilized.

1. Introduction

Bacteria-associated keratitis can cause vision threatening infections to the cornea [1]. In order to prevent the loss of vision, an eye care practitioner must identify the microorganism(s) causing the infection, determine the antibiotic susceptibility and recommend the best course of treatment before the infection causes significant damage. In severe cases, particularly those affecting the central cornea, it is customary to culture a corneal scrape [2] and, if the patient is a contact lens (CL) wearer, the lenses and samples from the patient's lens case are sometimes assessed for microbial contamination. However, culturing the bacteria from such samples can be challenging, and numerous studies have shown less than 70% success in obtaining positive cultures from corneal ulcers [3,4]. After antibiotic treatment has been initiated, it becomes increasingly difficult to identify the causative organism due to the inhibitory effect of the antibiotic on microbial growth [5]. Poor diagnosis and incorrect treatment can prolong infection and cause vision loss [6,7].

CL wearers have an increased risk of developing microbial keratitis (MK) compared to non-CL wearers [6,8,9]. In CL wearers, the incidence of MK ranges from 2.44 to 4.2 per 10,000 for all lens wearers, to as high as 21.8 per 10,000 for wearers who sleep in their lenses overnight [10,11]. One of the factors strongly associated with MK in CL wearers is the concurrent use of contaminated lens cases. CL cases are frequently contaminated with microorganisms, and the CL may act as a vehicle to transfer these pathogens to the eye [12–16]. There is a great diversity in the microbial species that can contaminate CL cases, including bacteria, yeast, mold, and

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Acanthamoeba [12,16–22]. There is also a wide variety of microorganisms that can cause MK, and of the bacterial species, 77 species from 42 different genera have been associated with MK [23]. Many of these species have been isolated as contaminants from CL cases [18,19,24].

Many studies have demonstrated that the most common species correlated with CL-associated MK is *Pseudomonas aeruginosa* [25–27]. More recently, three different species have been reported as additional bacteria that can cause MK in CL wearers. These Gram-negative bacteria are *Stenotrophomonas maltophilia*, *Elizabethkingia meningoseptica*, and *Delftia acidovorans*. CL-related infections with *S. maltophilia* have been reported as early as 1996 [28], *D. acidovorans* in 2001 [29], and *E. meningoseptica* in 2013 [30]. Additional infections with *S. maltophilia* have been reported to occur during corneal transplant surgeries and in co-infections with *Acanthamoeba* [31,32]. *E. meningoseptica* has also caused corneal infections in immunocompromised hosts [33]. These three microorganisms have been determined to be frequent contaminants in CL cases and their presence in the lens case is associated with the development of both corneal infiltrative events (CIE) and severe MK [13,18,34,35].

The presence of *S. maltophilia*, *E. meningoseptica*, and *D. acidovorans* in lens cases may be due to their ability to form biofilms [36–38]. A biofilm consists of a population of microorganisms that are adhered to a surface and are surrounded by a self-produced extracellular matrix [39]. Biofilms play a significant role in the resistance to disinfectants. Biofilm bacterial species exhibit different phenotypes compared to free-living bacteria, and resistant variants of the same bacterial species are more likely to be found in biofilms than planktonic microorganisms [40]. Nutrient, oxygen, and waste levels vary, according to the location of each bacterial cell in the biofilm [40,41].

To assess the presence of bacteria in CL cases and corneal infections, it is essential to choose the proper growth media and conditions. Tryptic soy agar (TSA) is a common media used for the recovery of microorganisms in disinfection efficacy studies [42]. Mueller–Hinton agar is used commonly in antibiotic susceptibility studies [43]. Other agars such as brain heart infusion (BHI), MacConkey, Columbia with 5% sheep's blood, and chocolate are used to recover bacteria from corneal ulcers [44,45]. The determination of the efficacy of disinfectants and antibiotics is dependent upon recovering viable bacteria. The use of inappropriate recovery media can cause a delay in determining the proper treatment for MK, leading to problematic outcomes [2].

The development of active biofilms by *S. maltophilia*, *E. meningoseptica*, and *D. acidovorans* on lens cases can be measured. The number of bacteria bound to the lens case surface can be enumerated by removing the bacteria using a calcium alginate swab and culturing on recovery media [46]. Also, the metabolic activity can be assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as an indirect measurement of determining the number of viable cells adhered to a lens case surface [47]. MTT is reduced by respiratory enzymes in the cell walls of bacteria and detection of the resulting reduced molecule, formazan, can be used to assess the viability of microbial biofilms [48].

In this study, the capability of *S. maltophilia*, *E. meningoseptica*, and *D. acidovorans* to form active biofilms in CL cases was analyzed. Also, growth of *S. maltophilia*, *E. meningoseptica*, and *D. acidovorans* on different media was assessed to determine the best recovery media for antimicrobial efficacy tests and culturing bacteria from corneal ulcers and CL cases of patients with CIE or MK.

2. Method

2.1. Growth of Stenotrophomonas maltophilia, Elizabethkingia meningoseptica, and Delftia acidovorans

The microorganisms were obtained from the American Type Culture Collection (ATCC Monassas, VA, USA). *S. maltophilia* (ATCC #17676, isolated from sinusitis pus), *E. meningoseptica* (ATCC #49470, isolated from a clinical specimen), and *D. acidovorans* (ATCC #17438, isolated from a pharyngeal swab) were grown on TSA (Becton, Dickinson, and Company, Sparks, MD, USA) at 37 °C for 24 h (*E. meningoseptica* and *D. acidovorans*) or 48 h (*S. maltophilia*). Microorganisms were then harvested from the TSA plates. Ten milliliters of phosphate-buffered saline (PBS) (Lonza Walkersville, MD, USA) was added to the TSA plate. A sterile plastic inoculating loop was used to suspend the culture, and the solution was transferred to a 10 mL centrifuge tube. The suspension was centrifuged at 490 RCF for 5 min. The supernatant was pipetted and disposed of, and the remaining pellet was suspended in 9 mL PBS, and mixed using a vortex. The solution was transferred into a spectrometer tube, and PBS was added until the desired spectrometer reading of 1.0 OD was obtained. This process was used for all three microorganisms.

2.2. Assessment of growth via recovery media

To assess the optimal media for the growth of each microorganism, six different growth media were selected. The media are described in Table 1 [49,50]. *S. maltophilia*, *E. meningoseptica*, and *D. acidovorans* were harvested and standardized to 1.0 OD. The standardized organism solution was then

Table 1
Media used for the recovery of *Stenotrophomonas maltophilia, Elizabethkingia meningoseptica,* and *Delftia acidovorans.*

Name	Manufacturer	Type	Common uses
MacConkey agar without crystal violet or salt	Becton, Dickinson, and Company, Sparks, MD, USA	Differential medium	Used for the isolation and differentiation of Gram-negative enteric bacilli.
Mueller-Hinton agar	Becton, Dickinson, and Company, Sparks, MD, USA	All purpose growth medium	Antimicrobial susceptibility testing of aerobic microorganisms.
Chocolate agar	Oxoid, Nepean, ON, Canada	All purpose growth medium	Growth of all species, especially fastidious microorganisms. Used as a primary isolation growth medium of clinical specimens.
Columbia agar with 5% sheep's blood	Oxoid, Nepean, ON, Canada	All purpose growth medium: high nutrition level	Growth of all species (nonfastidious and fastidious). Used as a primary isolation growth medium of clinical specimens.
Brain heart infusion agar	Becton, Dickinson, and Company, Sparks, MD, USA	All purpose growth medium: high nutrition level	Growth of many species, (nonfastidious and fastidious) including bacterial, yeast and molds.
Tryptic soy agar	Becton, Dickinson, and Company, Sparks, MD, USA	All purpose growth medium	Growth of nonfastidious microorganisms. Used in disinfection efficacy studies.