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ORIGINAL ARTICLE

Effects of lactoferricin B against keratitis-associated fungal biofilms

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Abstract Biofilms are considered as the most important developmental characteristics in ocular infections. Biofilm eradication is a major challenge today to overcome the incidence of drug resistance. This report demonstrates the in vitro ability of biofilm formation on contact lens by three common keratitis-associated fungal pathogens, namely, *Aspergillus fumigatus*, *Fusarium solani*, and *Candida albicans*. Antifungal sensitivity testing performed for both planktonic cells and biofilm revealed the sessile phenotype to be resistant at MIC levels for the planktonic cells and also at higher concentrations. A prototype lens care solution was also found to be partially effective in eradication of the mature biofilm from contact lenses. Lactoferricin B (Lacf, 64 μg/ml), an antimicrobial peptide, exhibited almost no effect on the sessile phenotype. However, the combinatory effect of Lacf with antifungals against planktonic cells and biofilms of three fungal strains that were isolated from keratitis patients exhibited a reduction of antifungal dose more than eightfold. Furthermore, the effect of Lacf in lens care solution against biofilms in which those strains formed was eradicated successfully. These results suggest that lactoferricin B could be a promising candidate for clinical use in improving biofilm susceptibility to antifungals and also as an antibiofilm-antifungal additive in lens care solution.

Keywords Biofilm eradication · Contact lenses · Keratitis · Lactoferricin B

Abbreviations

AmpB	Amphotericin B
ConA	Concanavalin A
Fluc	Fluconazole
Lacf	Lactoferricin B
MBEC	Minimum biofilm eradication concentration
MIC	Minimum inhibitory concentration
RPMI 1640	Roswell Park Memorial Institute-1640 medium
RGP	Rigid gas permeable
CFU	Colony-forming unit
Vori	Voriconazole
XTT	2,3-bis(2-Methoxy-4-nitro-5-sulfo-phenyl)-2*H*-tetrazolium-5-carboxanilide

Introduction

Fungal keratitis accounts for more than 50% of all ocular mycoses. This disease constitutes one of the most important causes of ocular morbidity and visual loss in developing nations such as India with a 62.2% rural, predominantly agritarian population where filamentous fungi, particularly *Aspergillus* sp. and *Fusarium* sp., are the major offending organisms [1, 2]. Similarly, in temperate climatic conditions yeast-related infections, notably *Candida albicans*, form the most important etiological agents.

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Although trauma continues to be an important risk factor in pathogenesis of fungal keratitis, an overall increase in contact lens-related fungal keratitis has been noted worldwide, from 5% in the 1980s to 52% in the early 21st century, along with recent epidemics of *Fusarium* keratitis in contact lens wearers. An important pathogenic mechanism for contact lens-related fungal keratitis that is currently being discussed is their ability to form biofilms [3–7]. However, the influence of contact lens type and lens care solution on biofilm formation by the common pathogenic fungal strains implicated in causation of infective keratitis, and their antifungal susceptibility, are largely unknown.

Several studies have additionally focused on screening antifungal peptides having novel activities to address emerging antifungal resistance [8]. Numerous antifungal peptides have been isolated from insects, amphibians, mammals, bacteria [9], and plants [10]. Lactoferricin B (Lacf), which is one such antimicrobial peptide, is derived from a multifunctional milk protein, lactoferrin, after proteolysis by pepsin. Earlier investigations have established its role in antimicrobial, antiviral, and antitumor activity [11]. However, the antifungal effect of lactoferricin B has not been evaluated, particularly in relationship to eradication of fungal biofilm.

In this current study we have used three fungal strains—*Aspergillus fumigatus*, *Fusarium solani*, and *Candida albicans*—isolated from patients with keratitis [2] to compare the differences in their capabilities of biofilm formation on contact lenses. We also evaluated the influence of addition of a novel antimicrobial peptide, lactoferricin B, on antifungal sensitivity for fungal biofilm and in combination with lens care solution to eradicate biofilm formation on contact lens.

Materials and methods

Fungal strains and growth conditions

Aspergillus fumigatus JM3, *Fusarium solani* JW21, and *Candida albicans* SJ11 were used in this study. All the strains were isolated from patients associated with fungal keratitis and characterized earlier [2]. Fungal species were collected from mature solid medium culture plate (Sabouraud dextrose agar) and mixed with liquid RPMI 1640 (Himedia, India) and incubated for 24 h at 30°C to obtain the relevant turbidity of 0.5×10^4 CFU/ml.

Contact lens and lens care solution

A silicon hydrogel lens made of senofilcon A (Acuvue Oasys, Johnson & Johnson) with 38% water content and a diameter of 14.5 mm was used. A prototype multipurpose lens solution (Complete Moisture Plus; Advanced Medical Optics) consisting of purified water, sodium chloride, potassium chloride, sodium phosphate dibasic, Poloxamer 237 (0.05% disodium), and sodium phosphate monobasic (monohydrate) with polyhexamethylene (0.0001%) as preservative was used to test biofilm eradication ability. Lens care solutions were stored at room temperature as per the manufacturer's suggestions.

Biofilm formation on contact lenses

To evaluate biofilm formation by *Aspergillus fumigatus*, *Fusarium solani*, and *Candida albicans* isolates, the soft contact lenses were separately submerged in six wells of flat-bottomed polystyrene plates containing 2 ml RPMI 1640 medium, inoculated with the respective fungal strain as a inoculum dose of 3.5×10^6 CFU/ml, and incubated for 72 h at 30°C. After 72 h, the lenses were removed and the planktonic cells were washed gently with 1× phosphate-buffered saline (PBS) buffer repeatedly. The lenses were then incubated at 37°C for 45 min in 4 ml PBS containing the fluorescent stain, concanavalin A-Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) conjugate (ConA) (200 μg/ml). ConA binds to glucose and mannose residues of fungal cell wall polysaccharides and emits green fluorescence [12]. After incubation with the dyes, the lenses were flipped and stained biofilms were visualized by fluorescence microscopy to compare their gross morphologies. Live–dead analysis was performed with BacLight Live/Dead dye (Invitrogen). The images were captured from the stained biofilms under a fluorescence microscope (Olympus IX 51, fitted with Evolution VF CCD camera).

XTT-reduction assay for quantification of biofilm

A semiquantitative measurement of biofilm formation was calculated using an XTT-reduction assay, adapted from previous reports [13, 14]. Briefly, XTT (2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2*H*-tetrazolium-5-carboxanilide; Sigma) was prepared in a saturated solution at 0.5 g/l in Ringer's lactate. Before each assay, an aliquot of stock XTT was thawed, and menadione (Sigma; 10 mM prepared in acetone) was added to a final concentration of 1 mM. A 100-ml aliquot of the XTT-menadione solution was then added to each prewashed biofilm containing lenses in an Eppendorf test tube (1.5 ml). Control wells for the measurement of background XTT-reduction levels were also used. The tubes were then incubated in the dark for up to 3 h at 37°C; subsequently the solutions were transferred from the tubes to 96-well plates. A colorimetric change in the XTT reduction was quantified by measuring OD_{490} using a Multiskan Spectrum-1500 spectrophotometer (Thermo