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Full Length Research Paper

Adherence and biofilm formation of *Fusarium oxysporum* isolated from a corneal ulcer

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Fusarium oxysporum is one of the principal agents of fungal keratitis, causing morbidity and loss of vision moreover contact lenses are important cofactor. This study aimed to evaluate the capacity for adherence, invasion, and biofilm formation in vitro of a sample of *Fusarium* isolated from a human corneal ulcer. The biofilm formation capacity of the fungal isolate was evaluated by the XTT reduction colorimetric method, on polystyrene Microplates at intervals of 1, 2, 4, 8, 24, and 48 hours. The viability of the adhered fungal cells was evaluated by LIVE/DEAD staining in confocal scanning fluorescence microscopy. Hydrophilic contact lenses were inoculated with 10^6 colony-forming units per milliliter of the fungus to evaluate adherence, by counting of strongly adhered cells, and the capacity for invasion observed by scanning electron microscopy. The formation of a biofilm by *F. oxysporum* was demonstrated by an increase in the intensity of XTT from 8 to 48 hours. LIVE/DEAD staining and XTT showed that the adhered fungi remained viable and metabolically active. *F. oxysporum* showed a high adherence capacity within 1 hour of incubation. After 72 hours the fungus had invaded and completely penetrated the contact-lens matrix. Concluding *F. oxysporum*, isolated from a fungal keratitis, showed a high capacity to adhere, invade, and form a biofilm. In addition, fungal cells remain viable and metabolically active after adhering, suggesting a potential for infection.

Key-words: Fungal keratitis, *Fusarium* biofilm, Contact lenses, Virulence factors.

INTRODUCTION

Therapeutic contact lenses (CL) have been extensively used as an alternative for vision correction, however,

their use is considered a predisposing factor for the development of keratitis (Klotz et al., 2000; Thomas, 2003; Yildiz et al., 2012). CL-related keratitis are well known, but clinically they are presumed as bacterial corneal ulcers (Willcox and Holden, 2001; Yildiz, et al. 2012). Nevertheless fungal corneal ulcer has assumed an important role in ophthalmology, since fungi also can

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affect the cornea as well as the eyelids, tear ducts, conjunctiva, sclera, and intra-ocular structures (Alfonso et al., 2006).

Several genera of fungi may be involved, vary according to climate, geographical conditions or the habits of the population (Thomas, 2003). The more implicated filamentous fungi are *Fusarium*, *Aspergillus*, *Cladosporium*, *Curvularia*, and *Penicillium* (Thomas, 2003; Nath et al., 2011). Among these, species of the genus *Fusarium* have been most often reported as causing fungal keratitis (Gopinathan et al., 2002; Pérez-Balbuena et al., 2009).

Usually, the fungi do not penetrate into the intact cornea, however, certain factors can compromise the corneal epithelium and contribute to the occurrence of infection, such as trauma caused by plants, surgery, pre-existing eye diseases, systemic illnesses, and the prolonged use of corticosteroids or topical antibacterial (Chaumeil et al., 2007), highlighting the use of CL and their improper cleaning (Dyavaiah et al., 2007). Changes in the normal conditions of the eye surface allow the microorganism access to the stream, where it multiplies and begins the infectious process (Sharma, 2001). Thus, prolonged use of CL, inadequate cleaning, epithelial trauma, hypoxia, and the use of topical corticosteroids are some of the situations that compromise the defense mechanisms of the host's eye surface, increasing the risk of developing keratitis (Weissman and Mondino, 2002).

On the other hand, some fungal virulence factors also can contribute to the pathogenesis of the keratitis like occurs in other mycotic infections, such as production of exoenzymes and toxins, morphogenesis, adherence, invasion, and biofuel production (Thomas, 2003). Biofilms are complex microbiological ecosystems formed by cells irreversibly adhered to a surface, surrounded by a matrix of extracellular products (Donlan and Costerton, 2002). Biofilms have been extensively studied, particularly in *Candida* spp. because of their importance in infections associated with the use of medical devices (Kuhn et al., 2002).

Some authors have shown the ability in biofuel production by filamentous fungi (Marques-Calvo, 2000; Sionov et al., 2001; Ahearn et al., 2007; Dyavaiah et al., 2007; Imamura et al., 2008). Nowadays it is known that fungal hyphae can penetrate the surface of most types of lenses including CL (Willcox, 2013). Apparently following the adherence process, the microorganism can form a biofilm on the CL surface, increasing the risk of developing keratitis (Klotz et al., 2000; Weissman and Mondino, 2002) but how this occurs is not yet fully elucidated. In this context, the objective of the present study was to evaluate the capacity for adherence, invasion, and biofilm formation of a sample of *Fusarium oxysporum* recently isolated from a human corneal ulcer.

MATERIAL AND METHODS

A sample of *F. oxysporum* recently obtained from a post-traumatic corneal ulcer of a patient seen at the Eye Hospital in Londrina, Paraná, Brazil, was used.

To prepare the inoculum, *F. oxysporum* was cultured in tubes containing Potato Dextrose Agar – PDA (Difco, USA) and incubated at 35°C for the first 48 h and then at 25°C until seven days of culturing were completed. The surface of the fungal colonies was gently washed with a 0.85% sterile physiologic saline solution (PSS). The fungal structures contained in the suspension were washed with PSS by centrifugation. The concentration of the inoculum was determined by spectrophotometry (Q.I. 108D Quimis, São Paulo, Brazil) and confirmed by colony-forming units (CFU) in Sabouraud Dextrose Agar (SDA) (Difco, USA) to comprise 10^6 CFU/ml.

The kinetics of *F. The oxysporum* biofilm formation was determined by the technique of reduction of tetrazolium salts (Ramage et al., 2001) using 2,3-bi's (2-methoxy-4-Nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) and phenazine methosulfate (PMS). Biofilm formation was evaluated in 96-well tissue culture polystyrene Microplates (Corning, NY, USA). An aliquot of 100 µL of Sabouraud Broth (Difco, USA) enriched with 8% glucose (Sigma Chemical Co., USA) (SDB) and 100 µL of the standardized microorganism suspension were pipetted into the test wells. A volume of 200 µL of SDB was inoculated into the control wells. The Microplates were incubated at 35°C for periods of 1, 2, 4, 8, 24, and 48 hours. The culture medium was removed, and the biofilm formed was washed two times carefully with sterile deionized water to remove the non-adhered fungi. Next, 100 µL of the solution containing 100 µg/ml of XTT (Sigma Chemical Co., USA) and 10 µg/ml of PMS (Sigma Chemical Co., USA) were added. The plates were incubated at 35°C for 3 h, protected from light. The absorbance of the calorimetric product was determined at 492 NM in a microplate reader (Expert Plus, Asys, Austria).

The viability of the fungal cells involved in adhesion and biofilm formation was determined using the fluorescent stain *LIVE/DEAD* (Molecular Probes, USA). The previously standardized fungal inoculum and an equal volume of SDB were placed on glass cover slips onto 6-well tissue culture microplate (Corning, NY, USA) and incubated at 35°C for 48 hours. The non-adhered cells were removed by successive washes, and *LIVE/DEAD* fluorescent stain was added to the biofilm adhered to the cover slip, which is a mixture of two fluorochromes, FUN-1 and calcofluor white at concentrations of 10 µM/ml and 25 µM/ml respectively. After 30 minutes of incubation at 30°C and protected from light, the biofilm was fixed in 5% paraformaldehyde (Sigma Chemical Co., USA) in

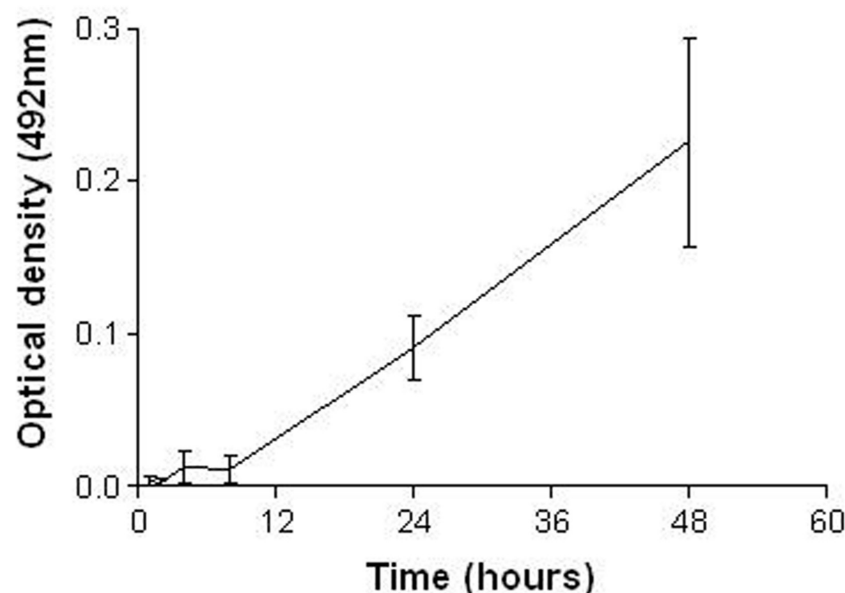


Figure 1. Kinetics of the metabolic activity of *F. oxysporum* in the formation of a biofilm on polystyrene microplates, determined by reduction of tetrazolium salts (XTT) over different periods of time.

phosphate buffered saline, pH 7.2. The cover slips were mounted on a slide, and the metabolically viable cells were observed with a confocal scanning fluorescence microscope (Zeiss LSM 5 Pascal, Germany).

The adherence capacity of *F. oxysporum* on hydrophilic CL with 66% water was determined by the technique of Sionov et al., (2001) with some modifications. On 6-Well Microplates (Corning, NY, USA) were added the standardized suspension of *F. oxysporum* and the CL, which were incubated at 35°C for 1 hour under continuous agitation at 120 RPM in a shaker (NT 712; Nova Técnica, Piracicaba, Brazil). The CL was transferred to another microplate, and the non-adhered fungi removed by successive washes. Adhered fungi were removed to the CL by 1 ml of 0.2% trypsin (Adolfo Lutz Institute, São Paulo, Brazil) with incubation for 30 minutes at 35°C under continuous agitation. Aliquots of 10 µL of this suspension were seeded onto Petri dishes containing SDA, for determination of the number of CFU of the strongly adhered fungi. The adherence capacity was expressed by the number of microorganisms adhered /mm² of CL surface.

The invasion capacity of *F. oxysporum* on hydrophilic CL with 36% water was evaluated by scanning electron microscopy (SEM). The biofilm was formed on the hydrophilic CL using the previously described methods, with incubation for 24 and 72 hours. CL was fixed in a

solution of 2.5% glutaraldehyde in 0.1 M cacodylate buffer (Sigma Chemical Co., USA). The whole CL was transversely sectioned and processed for documentation in a JEOL-JSM 6360 LV scanning electron microscope (Jeol Ltda, Tokyo, Japan) at the Electron Microscopy Center, Federal University of Paraná.

RESULTS

The kinetics of biofilm formation of *F. oxysporum* on the polystyrene surface is shown in Figure 1. In the first 8 hours of incubation there was a lag phase, followed by an exponential phase, indicating a linear increase in biofilm formation.

The fluorescent intravacuolar structures present in the interior of the fungal cells observed in the biofilm at 48 hours indicated that these cells were alive and metabolically active (Figures 2A-E).

It was also possible to demonstrate that *F. oxysporum* has an important capacity to adhere on CL. The CFU method showed that after 1 hour of co-incubation there were 27.03 ± 12.12 microorganisms adhered per square millimeter of CL surface.

The SEM analysis demonstrated that after 24 hours *F. oxysporum* was also capable of penetrating into the CL matrix (Figure 3). And after 72 hours, the fungal filaments

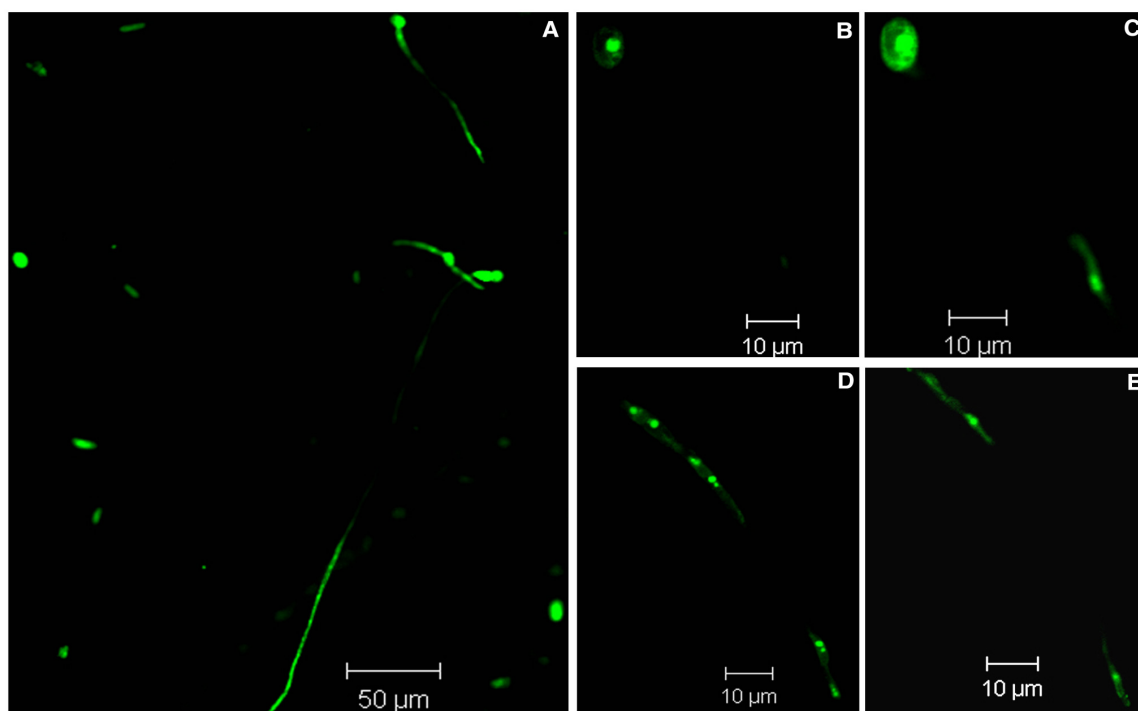


Figure 2. Fluorescent intravacuolar structures of *F. oxysporum* stained by *LIVE/DEAD*, observed by confocal fluorescence microscopy (A-E). The assay was done on a glass cover slip, after 48 hours of incubation at 35°C.

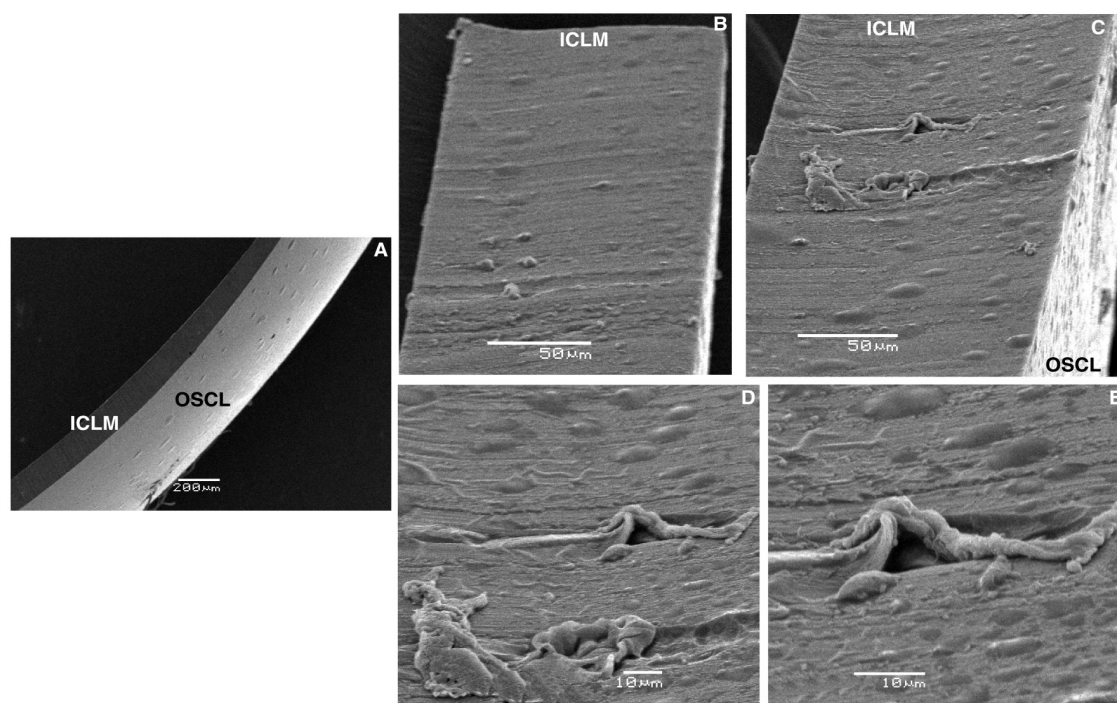


Figure 3. Scanning electron photomicrographs showing *F. oxysporum* intimately attached with the hydrophilic contact lens. Panoramic view of the sections, showing the regions observed (A). Fungal structures embedded in the contact lens matrix after 24 hours of incubation at 35°C, at different magnifications (B-E). OSCL: Outer Surface of the Contact Lens; ICLM: Interior of the Contact Lens Matrix

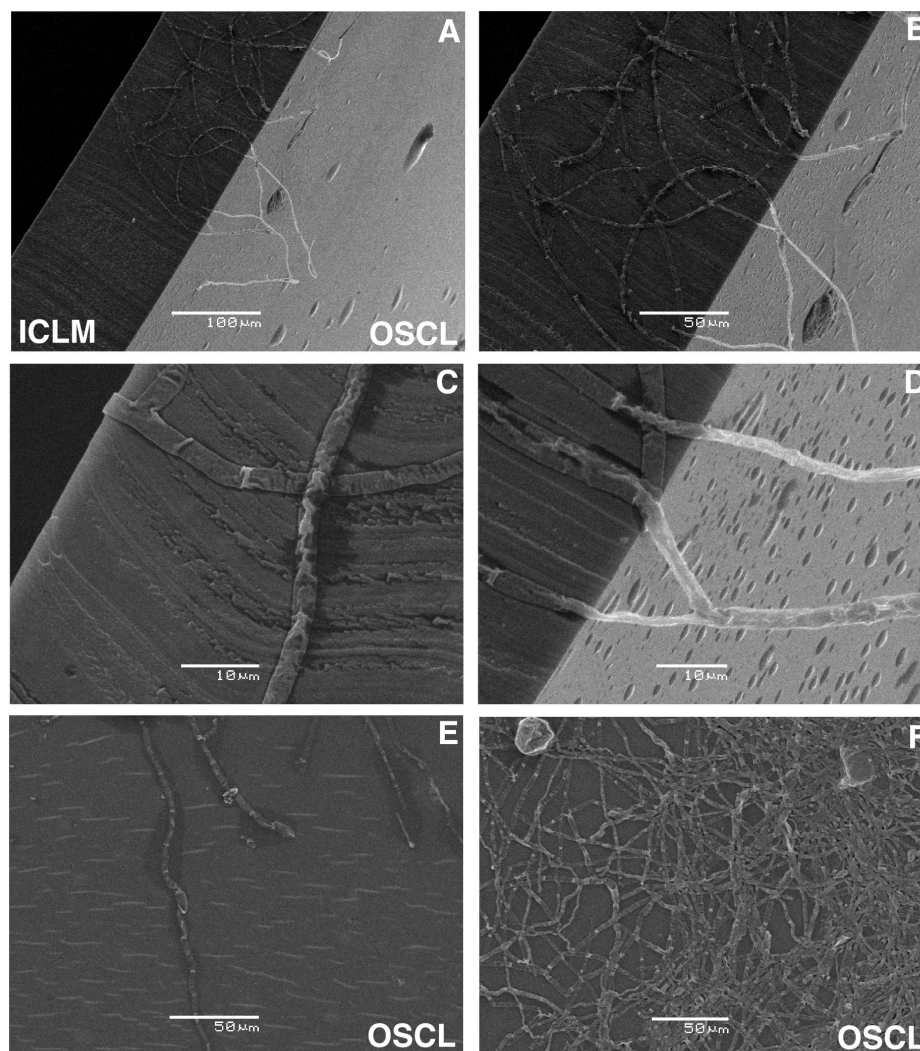


Figure 4. Scanning electron photomicrographs showing adherence and invasion of *F. oxysporum* in hydrophilic contact lenses after 72 hours of incubation at 35°C. Interior of the Contact Lens Matrix (ICLM): hyphae penetrating and invading completely the matrix (A-D). Strongly adhered hyphae and conidia (E) and the formation of a biofilm (F) were observed on the surface. OSCL: Outer Surface of the Contact Lens

had penetrated completely the CL, reaching the other side (Figures 4A-D). Analysis of the CL surface revealed an extensive network of strongly adhered fungal filaments, characterizing the mature biofilm (Figures 4E-F).

DISCUSSION

Current study proved that *F. oxysporum* has a potential to invade the CL by itself, and it should be involved in the pathogenesis of keratitis.

Figure 1 shows *F. oxysporum* is efficient in biofilm formation, showing an exponential curve of biofilm formation up to 48 hours of incubation. After this time, the exacerbated growth of the filament did not permit linear correlation, impeding precise determination of the time of stabilization of the biofilm, although it was possible to infer that this time was longer than the usual for *C. albicans* (Ramage et al., 2001).

Different systems and surfaces have been previously described for to evaluate fungal biofilm, according to the objective of each study (Tamura et al., 2003; Camacho et al., 2007; Vural et al., 2010). The colorimetric method,

based on the reduction of tetrazolium salts in Formosan derivatives, has been used in quantitative evaluation of biofilm formation (Ramage et al., 2001; Kuhn et al., 2002). This method makes possible to quantify the biomass produced and suggest that the fungus is alive, considering their metabolic activity. The results of the present study on a polystyrene microplate, which is a hydrophilic surface showed an increase in intensity of the XTT reaction after 8 hours reflecting the metabolic activity of *F. oxysporum* during the biofilm formation.

In sequence, through LIVE/DEAD stain (Figure 2) it was confirmed that *F. oxysporum* remains viable and metabolically active after adhering, reinforcing the results of the XTT reduction. These findings show that this fungus, even adhered and involved in the biofilm, is alive and therefore capable of causing infection.

Adherence capacity of *F. oxysporum* was also demonstrated using as surface a hydrophilic CL, the fungus was capable of adhering, invading, and completely penetrating into the CL matrix, as shown by SEM (Figures 3 and 4). According Marques-Calvo (2001) the fungal filament in fact invades and penetrates the matrix of CL, reaching into the cornea and resulting in infection.

These results are totally in accordance to Imamura et al. (2008), authors also showed that clinical isolates of *Fusarium* formed biofilms on all types of lenses tested and the biofilm architecture varied with the lens type. There is a consensus that fungal colonization on lens depends on factors related to its own composition, such as the amount of water and the kind of polymers. Data indicate that the fungi can better adhere and invade on CL with higher water content (Sionov et al., 2001). Nevertheless, it should be noted that the lens used for the SEM observations contained a small amount of water (36%). Even this composition permitted adherence and invasion by *F. oxysporum*, inferring that the adherence events could have been even more efficient if the patient using highly hydrated CL.

Among many fungal virulence factors, stands out the adherence capacity, which is a prerequisite for colonization and invasion, important events in the pathogenesis of infections. Considering that fungal colonization process begins with adhesion of fungal structures to the CL surface, and next there is the degradation of CL polymers by exoenzymes Marques-Calvo (2001), the data here showed allow to infer that colonization by *Fusarium* in contact lens represents a real risk factor for severe keratitis, which has been observed in the whole world (Ng et al., 2008; Kaufmann et al., 2008).

The current study also shows that *F. oxysporum* adhered and was efficient in form biofilm on different

kinds of material (polystyrene, glass, and hydrophilic CL), suggesting that this ability seems to be an intrinsic property of this microorganism. But it is important to note that the adherence potential of fungi is quite variable among species, even those belonging to the same genus (Sionov et al., 2001; Marques-Calvo, 2002). This study has evaluated *F. oxysporum* which is the specie more frequently isolated in our region (data no showed).

Another problem is keratitis caused by *Fusarium* has also been associated with the use of a multipurpose CL cleaning solution (Chang et al., 2006). According Ide et al. (2008) colonized lens may increase the risk of fungal keratitis and antifungal activity in contaminated multipurpose solutions may be insufficient to prevent fungal colonization of contact lens materials. Imamura et al. (2008) suggest that organizes in biofilm microorganisms would not be removed even by good hygiene practices. Recently Mela et al. (2014) have concluded that even with careful for handling, cleaning, and replacement of contact lens, the potential risk of contact lens-related fungal keratitis still remains.

In conclusion, this study has evaluated a recent isolate of *F. oxysporum* from fungal keratitis, which is one of the principal etiological agents of fungal keratitis. It was possible prove that *F. oxysporum* has a high capacity to adhere, invade, and form biofilm on CL. Furthermore, fungus maintains viability and metabolic activity after adhering, indicating appropriate potential to cause infection. Better understanding of the mechanisms of pathogenicity related to this microorganism in relation to some surfaces including CL may contribute to alerting doctors and patients for the prevention of fungal eye infections, because these are important factors in the virulence of the fungi and may be involved in their pathogenesis.

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