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Fungal biofilms in the hemodialysis environment

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ABSTRACT

Hemodialysis, which is a kidney failure treatment that uses hemodialysis machine, dialyzer, dialysis solution, catheters, and needles, favors biofilm formation. This study evaluates whether *Aspergillus*, *Candida,* and *Fusarium* can form biofilm in dialysis fluids. Biofilms were grown in 96-well microplates containing solutions (acid and basic) consisting of dialysate, dialysate *per se*, or dialysate plus glucose as culture medium. The biofilms were incubated at 30 °C for 72 h, quantified by the violet crystal methodology, and visualized by transmission electron microscopy. All the fungi formed biomass in all the tested solutions. However, Bonferroni analysis revealed that the dialysate facilitated *Aspergillus* biomass development, whereas the dialysate and dialysate with glucose provided similar *Fusarium oxysporum* biomass development. *Candida parapsilosis* development was favored in biofilms grown in basic electrolytic solution. Electron micrographs of biofilms that grew on catheters after 72 h showed that *Aspergillus* formed abundant hyphae; the extracellular matrix was visible on the surface of some hyphae when *Aspergillus* was grown in the dialysate. A multilayered hyphal structure emerged when *F. oxysporum* biofilms were incubated in the dialysate with glucose. *C. parapsilosis* biofilm growth in basic solution elicited a dense network of yeasts and pseudohyphae as well as the extracellular matrix; the biofilm was attached across the catheter length. This study may contribute to the formulation of new strategies to monitor biofilm formation and to increase knowledge associated with fungal biofilms in the dialysis environment.

1. Introduction

Biofilm-associated infections in medical devices have become a growing complication of hospitalization and have led to increased morbidity and mortality. These infections prolong hospitalization, require more extensive diagnosis and treatment, and are associated with additional costs [1]. Microbial biofilms consist of communities of cells that are adhered to both biotic and abiotic surfaces or to each other. Such biofilms are embedded by a matrix of extracellular polymeric substances produced by the microorganisms themselves. This matrix increases the microorganism chance of survival in a given environment [2].

Examples of microorganism sources are: i) the patients themselves, through skin and mucous membranes, infection foci at a distance, or bacteremias; ii) the healthcare professionals' hands while they handle patients during medical procedures; and iii) contaminated environment, water, and antiseptics [3,4]. The medical devices that are most affected by biofilm emergence include catheters of orthopedic, cardiac, and vascular prostheses [5] and of the vascular, urinary, and nervous system as well as hemodialysis machines [6,7].

Concerning hemodialysis, treated water, hydraulic pipes, circuits inside dialysis machines, and fluids used during hemodialysis, especially carbonated fluids and dialysate, constitute the most frequent contamination sites [8,9]. Factors such as temperature, electrolyte and glucose concentration, and presence of stagnation zones increase the likelihood that dialysis machines become contaminated [6,8,9].

When microbial metabolites or fragments present in the dialysate come into contact with the patient's blood during hemodialysis, they can stimulate membrane-bound leukocytes to circulate and induce human mononuclear cells to produce cytokines like interleukin-1 (IL-1) and tumor necrosis factor (TNF), which causes chronic microinflammation in hemodialysis patients or even death [10–12].

Bacteria have been the most associated with hemodialysis system contamination episodes [13–18]. However, studies dealing with fungal contamination are scarce [19–21].

In Latin America, increased population of immunocompromised patients and widespread use of chemotherapy and broad-spectrum antibiotics have raised the number of fungal infections caused by

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opportunistic fungi over the last two decades. *Candida* species are the most common fungal pathogens, followed by *Aspergillus, Fusarium*, and mucormycosis agents [22].

Candida biofilms are usual in clinical settings [23], so patients that require hemodialysis or peritoneal dialysis can be infected with *Candida* species [24]. *C. parapsilosis* has been reported as the second or the third most frequent pathogen among *Candida* species isolated from blood cultures in Europe, Canada, Asia, and Latin America [22,23,25]. *C. parapsilosis* can form biofilm in intravascular devices and prostheses, grow in hyperalimentation solution, and colonize the human hand, which all favor its survival and dissemination in the hospital environment [23].

Aspergillus and *Fusarium* are filamentous mould that can cause a broad spectrum of infections, including superficial, locally invasive, and disseminated infections. They are ubiquitous in the environment, and their conidia are present in air, water, and soil [26]. Among the *Fusarium* species associated with human infections, *F. solani* is the most common, followed by *F. oxysporum, F. verticillioidis*, and *F. moniliforme* [27]. Lass-Flörl et al. [28] analyzed 2071 lung biopsies from immunocompromised patients, to find that *Aspergillus fumigatus* prevailed, followed by *A. terreus, A. flavus,* and other *Aspergillus* species. In addition, Neely and Orloff [29] reported that *Aspergillus, Fusarium, Mucor*, and *Paecilomyces* species conidia survived for over 30 days, 10 days, over 20 days, and 11 days in hospital waste, respectively. We have already recovered yeast isolates (prevalence of *C. parapsilosis*) and filamentous fungi (predominance of *Fusarium* spp. followed by *Aspergillus* spp.) from i) water samples treated by reverse osmosis and distributed through the hydraulic circuit, ii) hemodialysis machine dialysate that was considered suitable for patients that were about to start a dialysis session, and iii) dialysis reuse waters [20,21].

In tropical countries, hot and humid climate, poor hygiene, and frequent use of broad-spectrum antibiotic therapy contribute the most to the risk of fungal colonization in chronic kidney disease (CKD) patients. Disease development is related to renal disease stage, type of therapy, and environmental fungal microbiota [30]. Here, we have evaluated whether *Aspergillus* spp., *Fusarium* spp., and *Candida parapsilosis* can form biofilm in fluids employed in hemodialysis (acid solution, basic solution, dialysate *per se*, and dialysate added with glucose 0.9% w/v) to improve our general understanding of the level of fungal infection risk associated with hemodialysis therapy.

2. Materials and methods

2.1. Fungal isolates

A total of 18 *Aspergillus* spp. isolates (A5, A16, A46, A70, A105, A109, A110, A171, A174, A181, A186, A189, A194, A223, A224, A226, A245, and A249), 10 *Fusarium* spp. isolates (F16, F24, F28, F30, F39, F43, F97, F157, F158, and F162), and 10 C. *parapsilosis* isolates (C3, C4, C8, C10, C14, C16, C17, C24, C82, and C83) previously recovered from the hydraulic circuit, the hemodialysis machine cannulae, and the reuse water of a Hospital Hemodialysis Center were included in this study. The filamentous fungi isolates were identified on the basis of slide culture and microscopic characterization as previously described [31–34]. The *C. parapsilosis* isolates had been previously identified by our group [21] with the aid of *Ban*I PCR RFLP of the SADH gene, as published by Tavanti et al. [35]. The fungi used in this study are part of the culture collection of the University of Franca, SP, Brazil.

2.2. Dialysis solutions

Both the acid electrolytic solution (NaCl – 172.20 g/l, KCl – 5.5 g/l, $CaCl_2.2H_2O$ – 8.0 g/l, and $MgCl_2.2H_2O$ – 3.72 g/l; final pH ranging from 1.7 to 2.6) and the basic solution (NaCl – 23.6 g/l and $NaHCO_3$ – 65.9 g/l; final pH ranging from 7.5 to 8.2) were prepared in the laboratory with high-purity salts. To prepare each liter of dialysate, 27 ml of the acid solution, 49 ml of the basic solution, and 924 ml of distilled water were mixed. All the solutions were submitted to autoclave sterilization at 121 °C for 15 min. According to Burmeister et al. [36], addition of glucose 0.09 g/l (w/v) to the dialysate prevents hypoglycemia episodes in patients during dialysis sessions. Thus, a dialysate solution added with glucose 0.09 g/l was prepared and filtered through a 22-μm-diameter pore filter (TPP, Biogen, Europe).

2.3. Biofilm formation

Fungal isolates were grown at 30 °C for seven days on potato dextrose agar (Difco Labs, MD, USA). Then, conidia were collected and diluted in each dialysis solution (acid solution, basic solution, dialysate, and dialysate added with glucose). The suspension was adjusted to turbidity 0.5 of the MacFarland standard (1.0×10^6 cells/ml). A conidial suspension in Roswell Park Memorial Institute (RPMI 1640, Sigma, St. Louis, MO) medium supplemented with L-glutamine, buffered with morpholinopropanesulfonic acid 0.165 M (MOPS, Sigma) to pH 7, and added with glucose 20 g/l was prepared to validate the experiments in dialysis fluids. Fungal biofilms were formed in flat-bottom polypropylene 96-well microtiter plates (Sigma Inc., SP, Brazil) and incubated at 30 °C for 72 h. The formed biomass was quantified. Experiments were performed in triplicate and on three different occasions.

2.4. Biofilm biomass determination

The crystal violet methodology described by Pathak et al. [37] was followed. Biofilms were quantified on a plate reader (Asys - Eugendorf, Salzburg, Austria); optical density was measured at 570 nm. Non-inoculated wells were used for background correction.

2.5. Sample preparation for scanning electron microscopy (SEM)

Biofilm formation on catheters was assessed by means of a 12-well microplate model as described by Chandra et al. [38]. Representative fungal biofilms were formed on polytetrafluorethylene (PTFE) catheter segments placed in 12-well tissue culture plates (TPP) containing 4 ml of fungal suspension at 1×10^6 cells/ml in RPMI 1640 broth and incubated at 35 ± 1 °C for 90 min without agitation to allow adhesion. Next, the fungal inoculum was removed, and the dialysis fluid that allowed better development of each tested fungal genus was added. The microplates were incubated for additional 72 h at 35 ± 1 °C. The resulting biofilms were rinsed with potassium phosphate buffer (PPB, 0.1 M, pH 7.2–7.4) and fixed during 1 h (2.5% glutaraldehyde, in PPB). Then, they were washed with PPB and dehydrated with a series of ethanol washes (15, 30, 50, 75, 95, and 100%), which was followed by drying. The catheters were sectioned with a scalpel, and the samples were imaged in a scanning electron microscope. All the assays were carried out in triplicate.

2.6. Statistical analysis

Graph construction and statistical analysis were conducted with the software GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA). Groups were described by the mean and standard deviation. Media and microorganisms were compared by means of a factorial analysis of variance (ANOVA) with repeated measures followed by the post-hoc Bonferroni test. Statistical significance was set at $p < 0.05$.

3. Results

Analysis of the colonies and micromorphological characteristics of the filamentous fungi showed that the *Aspergillus* isolates corresponded to *A. niger* (Supplementary Fig. 1A) and *A. terreus* (Supplementary Fig. 1B), and that *Fusarium* consisted of a single species, *F. oxysporum*