


Fungi in dialysis water and dialysate: occurrence, susceptibility to antifungal agents and biofilm production capacity

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ABSTRACT

The aim of this study was to investigate the occurrence of fungi in dialysis water and dialysate, in addition to evaluating the susceptibility to antifungals and the biofilm production capacity of isolated microorganisms. The samples were collected in three hemodialysis units in Bauru (Brazil), every 15 days (July 2017–June 2018) at post-reverse osmosis, reuse, and dialysate points. The fungi were isolated by spread plate on Sabouraud dextrose agar. Filamentous fungi were phenotypically identified and yeasts were subjected to molecular evaluation of the ITS region. Susceptibility test to antifungals was carried out by the broth microdilution method and biofilm production capacity was evaluated in microtiter plates using crystal violet staining. Fungi were isolated in 52/216 (24.1%) samples, with an average count of 16.3 (10–40) CFU/mL. Overall, 61 microorganisms were identified, with 54 (88.5%) filamentous fungi and 7 (11.5%) yeasts. The main genera included were *Penicillium*, *Cladosporium*, *Scedosporium*, *Rhinochloidiella*, *Fusarium*, and *Emmonsia*. Most isolates showed high values of minimum inhibitory concentration for 5-flucytosine and fluconazole and 35/45 (77.8%) isolates were classified as strong producers of biofilm. In order to increase the safety of the dialysis process, the adoption of control measures and monitoring of fungi in hemodialysis fluids is suggested.

Key words: antifungal susceptibility, biofilm, dialysate, dialysis water, fungi, hemodialysis

HIGHLIGHTS

- Fungi with pathogenic potential in hemodialysis fluids.
- Fungi with low susceptibility to antifungals.
- Fungi capable of producing biofilm.

INTRODUCTION

Chronic renal failure (CRF) is currently an important public health problem worldwide. It is considered a complex and multifactorial disease and has high rates of morbidity and mortality in several countries (Ashby *et al.* 2019).

Hemodialysis is the main form of treatment for CRF and, in general, is performed three times a week, in sessions that last about 3–4 hours, depending on the patient's clinical condition. In this therapy, water is used in the production of dialysate and also in the reuse of dialyzers, so that, during the hemodialysis sessions, patients are exposed, through the dialyzer's semi-permeable membrane, to approximately 400 liters of water per week. Therefore, the quality of the water used in this process is extremely important to avoid additional risks to patients' health (Totaro *et al.* 2017).

There are several guidelines that establish water quality standards for hemodialysis around the globe; however, except for some countries, such as Italy, Germany, Austria, and Sweden, most international standards do not include the counting of filamentous fungi and yeasts among microbiological parameters (Ward 2009).

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Naturally ubiquitous and known as saprophytes, in recent decades, fungi have arisen as emerging opportunistic pathogens, causing different episodes of nosocomial infections, especially in immunocompromised individuals (Durán Graeff *et al.* 2017).

Currently, the constant failures in the treatment of fungal infections have called attention to the emergence and spread of resistance to available antifungals, providing an increase in the lethality of these infections (Perlin *et al.* 2017). In addition, the possible production of biofilms by fungi can represent an important virulence factor for these microorganisms, giving them greater protection against adverse environmental conditions and greater resistance to antimicrobial agents (Sardi *et al.* 2014).

Several studies have already pointed out that the water distribution system seems to be an important reservoir of filamentous fungi and yeasts, so water is one of the main ways of spreading these microorganisms in hospital environments (Mesquita-Rocha *et al.* 2013; Arroyo *et al.* 2020). However, there are still few studies about the occurrence of fungi in hemodialysis fluids. Thus, the aim of this study was to investigate the occurrence, distribution, and diversity of filamentous fungi and yeasts in dialysis water and dialysate samples, in addition to evaluating the susceptibility to antifungal agents and the biofilm production capacity of isolated microorganisms.

METHODS

Samples

Dialysis water and dialysate samples were collected from three hemodialysis units, called A, B, and C, located in the city of Bauru, State of São Paulo, Brazil. The collections were performed according to the recommendations of the *Standard Methods for the Examination of Water and Wastewater* (APHA 2012), every 15 days, from July 2017 to June 2018, in the following points of the water treatment and distribution systems: (i) right after the treatment (post-reverse osmosis), (ii) in the reprocessing room of the dialyzers (reuse), and (iii) in the dialysis machine (dialysate) – a total of 216 samples.

Fungal culture and identification

The isolation of fungi was performed by spread plate method on Sabouraud dextrose agar (SDA) supplemented with chloramphenicol (0.05 g/L), and incubated at 25 °C/15 days (APHA 2012). Filamentous fungi were phenotypically identified at the gender level through the study of their macroscopic characteristics (giant colony technique) and microscopic characteristics (microculture test). Yeasts were analyzed for their macroscopic, microscopic, reproductive, and physiological characteristics, and afterwards, they were subjected to molecular evaluation of the ITS (internal transcribed spacer) region of the rDNA (White *et al.* 1990), for confirmation of species.

DNA was extracted using PrepMan™ Ultra Sample Preparation (Applied Biosystems), according to the manufacturer's recommendations and quantification in a spectrophotometer (Epoch-Biotek, Winooski, VT, USA). The PCR reaction was carried out using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), under the following conditions: 0.2 µM each primer, 1.6 mM MgCl₂, 0.2 mM dNTP, reaction buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.0), 1 U Platinum® Taq DNA Polymerase (Invitrogen, USA), and 30 ng genomic DNA. Amplification was performed in Veriti thermo-cycler (Applied Biosystems, IL, USA), with a cycle of initial denaturation at 95 °C/5 min, followed by 30 cycles at 95 °C/50 sec, 50 °C/50 sec, 72 °C/50 sec, and final extension at 72 °C/5 min. The amplified product was analyzed by electrophoresis in 1.5% agarose gel and visualized under ultraviolet light (296 nm) by transilluminator (Syngene®, DigiGenius, MD, USA), and the image was captured by digital documentation system EOS utility® (Canon, USA). Amplicons were sequenced, the sense and antisense were visualized by using Chromas 2.3 software (Technelysium, Helensvale, Australia), aligned by the MEGAX software, submitted to BLASTn (<http://www.ncbi.nlm.nih.gov/BLAST>), and compared with the sequences deposited in the GenBank.

Susceptibility testing for antifungal agents

To determine the minimum inhibitory concentration (MIC), the broth microdilution method was used according to the recommendations of the European Committee on Antibiotic Susceptibility Testing (EUCAST) (Arendrup *et al.* 2014, 2015).

The tested antifungals included micafungin (0.0156 to 16 mg/L), caspofungin (0.0156 to 16 mg/L), amphotericin B (0.0313 to 16 mg/L), 5-flucytosine (0.125 to 64 mg/L), fluconazole (0.125 to 64 mg/L), itraconazole (0.0019 to 8 mg/L), voriconazole (0.0019 to 8 mg/L), and miconazole (0.0019 to 16 mg/L). Strains of *Candida parapsilosis* (ATCC 22,019), *Candida krusei* (ATCC 6,058), and *Aspergillus fumigatus* (ATCC 204,305) were used as quality controls.

Considering that there are no defined breakpoints for all the isolated fungi, the MIC values were interpreted according to the instructions available in the documents EUCAST E.Def 9.2 (Arendrup *et al.* 2014), EUCAST E.Def 7.3 (Arendrup *et al.* 2015), CLSI M38-A2 (CLSI 2008a), CLSI M27-A3 (CLSI 2008b), and CLSI M27-S4 (CLSI 2012).

Biofilm production test

The biofilm production capacity was evaluated in microtiter plates using the crystal violet staining method, according to Stepanović *et al.* (2007), with modifications for yeasts and filamentous fungi.

The filamentous fungi were grown on potato dextrose agar, at 25 °C. Then, for each isolate, a suspension in sterile saline with conidia or sporangiospores was prepared, adjusted in a spectrophotometer (Epoch-Biotek, Winooski, VT, USA) at a concentration of 1×10^4 CFU/mL. Then, 20 µL of the fungal suspension and 180 µL of tryptic soy broth (TSB), supplemented with 1% glucose, were incubated in plates at 25 °C/48 h. After incubation, the following steps were performed: washing with distilled water, fixing with methanol (150 µL/20 min), staining with violet crystal 2% (150 µL/15 min), washing with distilled water, resuspension with acetic acid 33% (150 µL/30 min), and absorbance reading in a spectrophotometer at 590 nm.

The yeasts were grown on Sabouraud dextrose agar at 36 °C/24–48 h and, based on the growth obtained, a suspension was prepared in 3 mL of sterile saline, according to the McFarland standard 4. Twenty µL of the suspension and 180 µL of Sabouraud broth were used, supplemented with 8% glucose, and the plate was incubated at 35 °C/24 h. After incubation, the following steps were performed: washing with distilled water, fixing with methanol (110 µL/15 min), staining with violet crystal 0.4% (110 µL/45 min), washing with distilled water, resuspension with acetic acid 33% (200 µL/30 sec), and absorbance reading in a spectrophotometer at 595 nm.

The tests were performed in quadruplicate and a white well (without inoculum, with culture medium only) was used to correct the absorbance value. After correction – considering the average optical density of the sample (OD) subtracted from the average of the optical density of the white (ODc) – the isolates were classified as strong producers ($OD > 4 \times ODc$), moderate producers ($2 \times ODc < OD \leq 4 \times ODc$), weak producers ($ODc < OD \leq 2 \times ODc$) or non-biofilm producers ($OD \leq ODc$). As a positive control, the reference strain *C. parapsilosis* (ATCC 22,019) was used.

Statistical analysis

Statistical analysis was performed with the aid of the software Statistica 10.0 (Statsoft, Tulsa, OK, USA, 2011), considering a significant difference when $p < 0.05$.

RESULTS

Fungi were isolated in 52/216 (24.1%) samples, with similar percentages of positivity in all units investigated, however, with significant predominance in samples from the reuse room and dialysate. Among the positive samples, the average (minimum–maximum) fungal count was 16.3 (10–40) CFU/mL. The highest count values were noted at unit C and at the post-reverse osmosis point, but without significant statistical differences (Table 1).

Table 1 | Occurrence of fungi in dialysis water and dialysate samples, according to the hemodialysis units and the collection points investigated

Origin of the samples	Positivity	P-value*	Count (CFU/mL) ^a	P-value**
Hemodialysis units				
A	20/72 (27.8%)	0.492	16.0 (10–40)	0.165
B	18/72 (25.0%)		12.8 (10–20)	
C	14/72 (19.4%)		21.4 (10–40)	
Collection points				
Post-reverse osmosis	9/72 (12.5%) b	0.008	23.3 (10–40)	0.069
Reuse	18/72 (25.0%) ab		13.9 (10–40)	
Dialysate	25/72 (34.7%) a		15.6 (10–40)	

^aData expressed on average (minimum–maximum).

*Chi-square test. Different letters indicate between which samples significant differences were observed after the Marascuilo method for multiple comparisons.

**Kruskal–Wallis test.

Among the 52 positive samples for fungi, 45 (86.5%) showed growth of filamentous fungi, 4 (7.7%) of yeasts, and 3 (5.8%) of filamentous fungi and yeasts concomitantly. Overall, 61 microorganisms were identified, with 54 (88.5%) filamentous fungi and 7 (11.5%) yeasts. The main genera included were *Penicillium*, *Cladosporium*, *Scedosporium*, *Rhinochadiella*, *Fusarium*, and *Emmonsia* (Figure 1). Table 2 shows the complete distribution of isolated microorganisms in relation to the hemodialysis units and the collection points investigated.

The susceptibility test to antifungal agents revealed several MIC values for all the analyzed drugs (Table 3). Most filamentous isolates showed high MIC values for 5-flucytosine and fluconazole. Some *Penicillium* isolates also showed low susceptibility to echinocandins and voriconazole. High MICs for caspofungin and miconazole were noted in microorganisms of the genera *Scedosporium* and *Emmonsia*, respectively. *Fusarium* spp. and *Rhinochadiella* spp. isolates showed low susceptibility to all tested drugs. Even for amphotericin B, some filamentous isolates exposed high MICs, with values ranging between 0.125 and >16 µg/mL. Among the yeasts, *Candida orthopsilosis* was sensitive to the tested antifungals and *Cryptococcus laurentii* showed high MIC values for echinocandins, 5-flucytosine, and fluconazole.

The analysis of the biofilm production capacity included 45 filamentous fungi and 3 yeasts (Table 4). All the filamentous fungi produced biofilm, so 35/45 (77.8%) isolates were classified as strong producers and 10/45 (22.2%) as moderate producers, with no statistically significant differences between the analyzed genera. There were also no significant differences between the filamentous isolates in terms of hemodialysis units and the collection points investigated (Table 5). Among the yeasts, 2/3 (66.7%) isolates were not able to produce biofilm and 1/3 (33.3%) showed a weakly producing profile.

The investigation of a possible correlation between susceptibility to antifungal agents and the biofilm production capacity of the filamentous isolates showed a positive correlation (despite the weak intensity: $0.3 < r < 0.5$) between the MIC values for micafungin, amphotericin B, and 5-flucytosine and the absorbance values of the biofilm production test (Table 6).

DISCUSSION

Water is essential in dialysis therapy and its physical–chemical and microbiological quality must be guaranteed (Totaro *et al.* 2017).

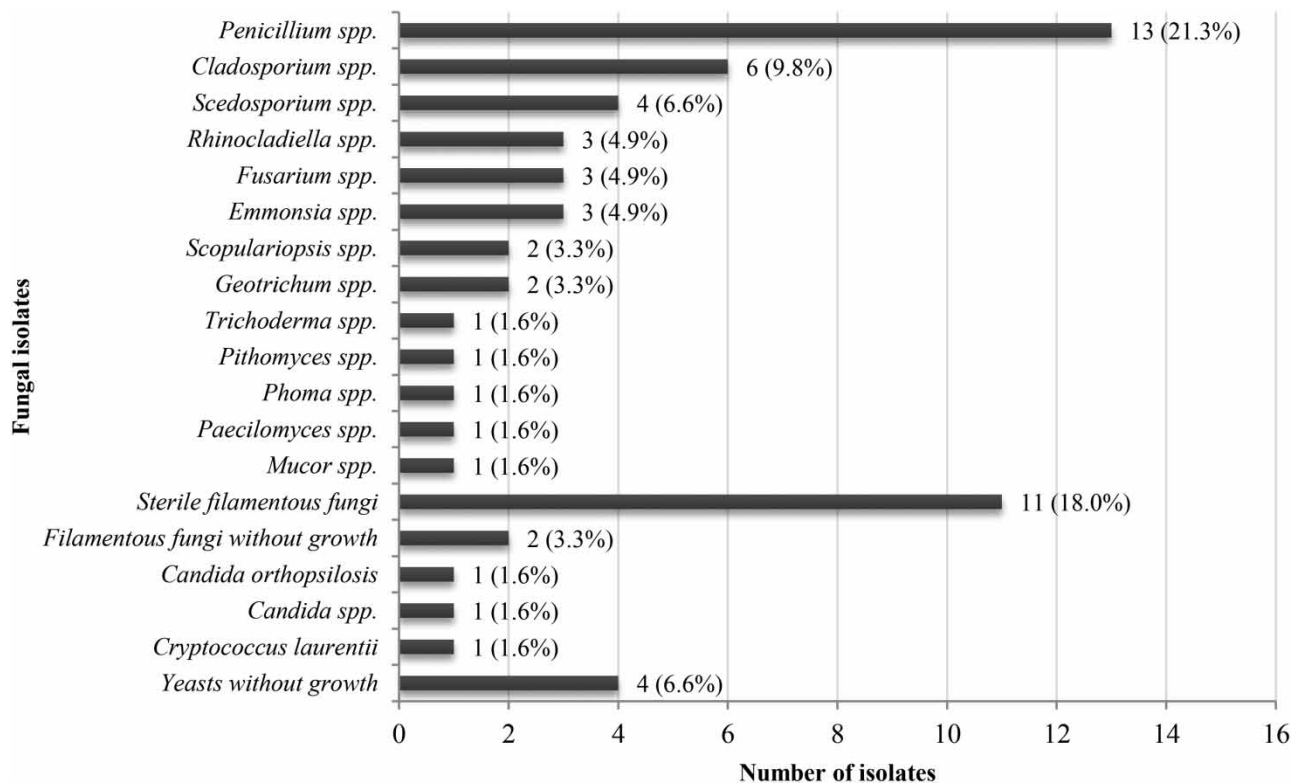


Figure 1 | Fungi isolated from dialysis water and dialysate samples, from three hemodialysis units, located in Bauru, State of São Paulo, Brazil.

Table 2 | Distribution of fungal isolates, according to the hemodialysis units and the collection points investigated

Fungal isolates	Hemodialysis units								
	A			B			C		
	Collection points			Collection points			Collection points		
	Post-reverse osmosis	Reuse	Dialysate	Post-reverse osmosis	Reuse	Dialysate	Post-reverse osmosis	Reuse	Dialysate
Filamentous fungi									
<i>Penicillium</i> spp.	3	2	1	0	0	1	0	2	4
<i>Cladosporium</i> spp.	1	2	0	0	1	1	0	0	1
<i>Scedosporium</i> spp.	0	1	0	0	2	0	1	0	0
<i>Emmonsia</i> spp.	0	0	1	0	0	1	1	0	0
<i>Fusarium</i> spp.	0	0	0	0	1	1	0	1	0
<i>Rhinochadiella</i> spp.	2	0	0	0	0	0	0	0	1
<i>Geotrichum</i> spp.	0	1	0	0	0	1	0	0	0
<i>Scopulariopsis</i> spp.	0	0	1	0	0	1	0	0	0
<i>Mucor</i> spp.	0	0	0	0	0	0	0	0	1
<i>Paecilomyces</i> spp.	0	0	0	1	0	0	0	0	0
<i>Phoma</i> spp.	0	1	0	0	0	0	0	0	0
<i>Pithomyces</i> spp.	0	0	0	0	0	0	1	0	0
<i>Trichoderma</i> spp.	0	0	0	0	0	1	0	0	0
Sterile filamentous fungi	0	1	3	0	2	3	1	0	1
Filamentous fungi without growth	0	1	0	1	0	0	0	0	0
Yeasts									
<i>Candida orthopsilosis</i>	0	0	0	0	0	0	0	0	1
<i>Candida</i> spp.	1	0	0	0	0	0	0	0	0
<i>Cryptococcus laurentii</i>	0	0	0	0	1	0	0	0	0
Yeasts without growth	0	0	1	0	0	1	0	1	1

In the present study, fungi were detected in 24.1% of the dialysis water and dialysate samples from three hemodialysis units in Bauru (Brazil), with an average (minimum–maximum) count of 16.3 (10–40) CFU/mL. A similar study undertaken in Greece found filamentous fungi and yeasts in 95.3% of dialysis water samples and in 90.6% of dialysate samples (Arvanitidou *et al.* 2000). In Iran, Mahmoudabadi *et al.* (2011) reported fungi in 27.4% of the analyzed samples, and in Italy, Schiavano *et al.* (2014) isolated these microorganisms in 12.1% of the samples from eight hemodialysis units, with counts that varied between 1 and 420 CFU/100 mL. In Brazil, other studies described fungal contamination in 66% (Figel *et al.* 2013) and 89% of the samples, of which, some showed >100 CFU/mL (Pires-Gonçalves *et al.* 2008). However, it is important to note that due to the diversity of methodologies used, with different isolation techniques, applied volumes, culture medium, and incubation periods, it is not always possible to make direct comparisons between studies.

Some authors have described a higher frequency and concentration of microorganisms in dialysate samples, suggesting that contamination and proliferation occur mainly in dialysis machines (Heidarieh *et al.* 2016). However, these findings were not so clear in our research, which showed a higher frequency of fungi in the reuse room and in the dialysate, without significant differences related to their concentrations in the different collection points, thus we reinforce the need for intensification of the maintenance, cleaning, and disinfection measures throughout the water treatment and distribution system.

Filamentous fungi were the main fungal contaminants isolated in this study. Similarly, other studies have also shown the predominance of these microorganisms, with emphasis on the genera *Cladosporium*, *Penicillium*, *Aspergillus*, *Fusarium*, *Trichoderma*, among others (Mahmoudabadi *et al.* 2011; Figel *et al.* 2013; Schiavano *et al.* 2014). Yeasts, including *Candida*

Table 3 | Susceptibility to antifungal agents

Fungal isolates	Antifungals							
	Minimum inhibitory concentration (µg/mL)							
	MICA	CPFG	AMFO	5-FC	FLC	ITZ	VCZ	MCZ
Filamentous fungi								
<i>Penicillium</i> spp. I	0.125	0.125	0.125	0.5	1	0.125	0.25	4
<i>Penicillium</i> spp. II	0.0625	1	2	8	>64	1	>8	2
<i>Penicillium</i> spp. III	1	0.5	0.5	0.5	2	1	0.5	0.5
<i>Penicillium</i> spp. IV	8	1	>16	16	>64	1	8	2
<i>Penicillium</i> spp. V	0.0156	4	0.5	0.125	>64	1	8	2
<i>Penicillium</i> spp. VI	0.0313	4	0.5	0.125	>64	8	>8	4
<i>Penicillium</i> spp. VII	>16	16	1	0.125	>64	>8	>8	>16
<i>Penicillium</i> spp. VIII	2	0.5	2	32	>64	2	>8	2
<i>Penicillium</i> spp. IX	>16	>16	2	8	>64	1	>8	1
<i>Penicillium</i> spp. X	>16	>16	4	8	>64	0.25	4	0.5
<i>Penicillium</i> spp. XI	>16	>16	2	64	>64	1	>8	1
<i>Penicillium</i> spp. XII	4	0.5	4	2	>64	8	>8	1
<i>Cladosporium</i> spp. I	0.5	0.5	1	16	8	4	0.5	>16
<i>Cladosporium</i> spp. II	0.25	0.5	1	0.25	8	>8	4	2
<i>Cladosporium</i> spp. III	1	2	1	0.5	0.5	0.0625	0.5	1
<i>Scedosporium</i> spp. I	1	8	0.25	64	64	0.25	1	1
<i>Scedosporium</i> spp. II	0.5	4	0.25	1	0.5	1	0.125	0.5
<i>Scedosporium</i> spp. III	4	2	0.5	>64	8	0.125	1	2
<i>Scedosporium</i> spp. IV	0.0625	4	0.5	>64	32	0.5	0.5	0.5
<i>Emmonsia</i> spp. I	0.125	0.25	8	8	8	>8	1	16
<i>Emmonsia</i> spp. II	0.5	0.25	>16	64	16	4	1	4
<i>Emmonsia</i> spp. III	1	1	1	16	1	0.125	0.0313	>16
<i>Fusarium</i> spp. I	16	4	1	>64	>64	2	8	4
<i>Fusarium</i> spp. II	8	4	4	>64	>64	>8	>8	16
<i>Rhinochadiella</i> spp. I	16	16	16	64	64	8	8	16
<i>Rhinochadiella</i> spp. II	4	16	>16	>64	>64	8	>8	2
<i>Rhinochadiella</i> spp. III	2	4	1	4	>64	2	4	2
<i>Scopulariopsis</i> spp.	0.0156	0.125	1	64	>64	0.25	1	2
<i>Paecilomyces</i> spp.	1	2	1	4	>64	2	>8	4
<i>Phoma</i> spp.	2	8	1	64	4	0.25	0.125	0.125
<i>Pithomyces</i> spp.	0.5	2	0.25	2	4	0.125	0.125	0.125
Yeasts								
<i>Candida orthopsilosis</i>	0.25	2	0.5	1	0.25	0.0313	0.0313	0.0625
<i>Cryptococcus laurentii</i>	>16	16	0.5	>64	4	0.125	0.125	0.5

MICA, micafungin; CPFG, caspofungin; AMFO, amphotericin B; 5-FC, 5-flucytosine; FLC, fluconazole; ITZ, itraconazole; VCZ, voriconazole; MCZ, miconazole.

spp., *Cryptococcus* spp., *Trichosporon* spp., and *Rhodotorula* spp., have also been reported in hemodialysis fluids, with different frequencies (Arvanitidou *et al.* 2000; Montanari *et al.* 2018).

The most frequent isolates in our research were members of the genus *Penicillium*, which have already been described in several cases of invasive fungal infections, with pulmonary, pericardial, hepatosplenic involvement, and others (Geltner *et al.*

Table 4 | General biofilm production capacity

Fungal isolates	Absorbance (nm) ^a	Classification (n)			
		Strong producer	Moderate producer	Weak producer	Non-producer
Filamentous fungi					
<i>Penicillium</i> spp.	1.92 (0.47–3.34)	11	2	0	0
<i>Cladosporium</i> spp.	1.69 (1.23–2.59)	4	0	0	0
<i>Scedosporium</i> spp.	2.40 (2.15–2.75)	4	0	0	0
<i>Emmonsia</i> spp.	2.78 (2.65–3.01)	1	2	0	0
<i>Fusarium</i> spp.	3.01 (2.92–3.09)	2	0	0	0
<i>Rhinochadiella</i> spp.	2.71 (2.28–2.94)	2	1	0	0
<i>Geotrichum</i> spp.	1.90 (1.20–2.60)	1	1	0	0
<i>Scopulariopsis</i> spp.	1.52 (1.23–1.80)	1	1	0	0
<i>Mucor</i> spp.	2.24	1	0	0	0
<i>Paecilomyces</i> spp.	2.32	1	0	0	0
<i>Phoma</i> spp.	2.03	1	0	0	0
<i>Pithomyces</i> spp.	1.49	0	1	0	0
Sterile filamentous fungi	2.03 (1.67–2.48)	6	2	0	0
P-value*	0.083	–	–	–	–
Overall	2.11 (0.47–3.34)	35 (77.8%)	10 (22.2%)	0	0
Yeasts					
<i>Candida orthopsilosis</i>	2.00	0	0	1	0
<i>Candida</i> spp.	1.72	0	0	0	1
<i>Cryptococcus laurentii</i>	1.63	0	0	0	1
Overall	1.78 (1.63–2.00)	0	0	1 (33.3%)	2 (66.7%)

^aData expressed on average (minimum–maximum).

*Kruskal–Wallis test.

Table 5 | Biofilm production capacity of filamentous fungal isolates, according to the hemodialysis units and the collection points investigated

Origin of the samples	Biofilm production capacity Absorbance (nm) ^a	P-value*
Hemodialysis units		
A (n = 19)	1.88 (0.47–3.01)	0.232
B (n = 12)	2.25 (1.67–2.92)	
C (n = 14)	2.29 (1.36–3.34)	
Collection points		
Post-reverse osmosis (n = 11)	2.06 (0.92–2.94)	0.462
Reuse (n = 14)	1.92 (0.47–3.09)	
Dialysate (n = 20)	2.26 (1.23–3.34)	

^aData expressed on average (minimum–maximum).

*Kruskal–Wallis test.

2013; Ramírez *et al.* 2018). *Cladosporium* spp. are among the most largely dispersed fungi in the environment and are commonly associated with allergic rhinitis and localized skin and subcutaneous lesions, but may additionally cause deeper and more widespread infections (Sandoval-Denis *et al.* 2015). The genus *Scedosporium* has at least ten species that are possibly

Table 6 | Correlation between susceptibility to antifungal agents and the biofilm production capacity of filamentous fungal isolates

Antifungals	Biofilm production capacity Spearman correlation (r)	P-value
Micafungin	0.437	0.012
Caspofungin	0.255	0.160
Amphotericin B	0.361	0.042
5-Flucytosine	0.480	0.005
Fluconazole	0.118	0.518
Itraconazole	0.177	0.334
Voriconazole	0.092	0.615
Miconazole	0.102	0.577

pathogenic to humans, capable of causing a wide spectrum of diseases (Chen *et al.* 2016). Frequently, invasive infections caused by *Scedosporium* spp. are difficult to treat and are associated with high rates of morbidity and mortality (Bernhardt *et al.* 2018). *Fusarium* spp. are among the filamentous fungi of greatest clinical importance (Arnoni *et al.* 2018) and, like *Penicillium* spp., they are important producers of mycotoxins, which can cause several kinds of acute and chronic pathological damage (Novak Babič *et al.* 2017). Microorganisms of the genera *Rhinoctadiella*, *Emmonsia*, *Scopulariopsis*, *Geotrichum*, *Trichoderma*, *Pithomyces*, *Phoma*, *Paecilomyces*, and *Mucor* have also been described in severe cases of invasive infections (Nucci *et al.* 2010; Schwartz *et al.* 2015; Durán Graeff *et al.* 2017; Garcia-Hermoso *et al.* 2018; Velasco & Revankar 2019).

Relating to the yeasts, they are certainly the main cause of nosocomial fungal infections. Invasive candidiasis is the most prevalent fungal disease among hospitalized patients and affects approximately 250,000 people, annually, throughout the world, with more than 50,000 reported deaths (Kullberg & Arendrup 2015). Although *C. albicans* remains the most involved species in pathogenic conditions, in the last decades there has been a significant change in the epidemiological profile of these infections, with an increase in cases caused by non-albicans *Candida*, including *C. orthopsilosis* (Pappas *et al.* 2016; Arastehfar *et al.* 2019), which was isolated in this study. *Cryptococcus laurentii* has also expanded as a causative agent of opportunistic infections in humans, with reports of pulmonary infections, meningitis, peritonitis, and fungemias (Smith *et al.* 2017).

Until now, little has been known about the clinical complications caused by fungal contamination of hemodialysis fluids, because there are few studies in the literature. Some episodes of fungemia by *Phialemonium curvatum* and *C. parapsilosis* have been described by some authors, related to inadequate reprocessing of dialyzers and reflux of residual fluids (Rao *et al.* 2009; Oyong *et al.* 2014).

In the last years, antifungal resistance has become an eminent global problem (Houšť *et al.* 2020). Corroborating with our findings, several studies have pointed out low activity of 5-flucytosine and fluconazole against filamentous fungi (CLSI 2008a; Peng *et al.* 2019). Commonly, *Scedosporium* spp. show *in vitro* resistance to several antifungal agents, including echinocandins, and *Fusarium* spp. are among the most drug-resistant fungi available (Tortorano *et al.* 2014). In fact, the emergence of several *Fusarium* species with a profile of resistance to multiple drugs has become a public health problem, with an unfavorable prognosis and high mortality rates (Taj-Aldeen 2017). Some special attention should be paid to *Rhinoctadiella* isolates which have shown little susceptibility to the antifungal agents used, unlike some studies that reported low MIC values for amphotericin B and azoles in general for these microorganisms (Nucci *et al.* 2001; Sun *et al.* 2019). Other studies involving yeasts isolated from hemodialysis fluids have also not shown resistance to antifungals among microorganisms of the *C. parapsilosis* complex (Pires *et al.* 2011); however, clinical failures in infections caused by *C. orthopsilosis* have been reported by some authors (Heslop *et al.* 2015; Charsizadeh *et al.* 2018). *Cryptococcus laurentii* with reduced susceptibility to some drugs has also been described in the literature (Ajesh & Sreejith 2012).

According to some authors, the expansion of antifungal use in hospital environments and in agriculture may be significantly influencing the emergence of more resistant fungi, with elevated MICs (Chowdhary *et al.* 2013; Jensen 2016).

Currently, the reference documents recommended by EUCAST and the Clinical and Laboratory Standards Institute (CLSI) establish breakpoints for some antifungals for only species of the genera *Candida* and *Aspergillus* (CLSI 2008a, 2008b, 2012;

Arendrup *et al.* 2014, 2015). Thus, for most emerging fungal pathogens there are still no defined breakpoints, which makes the interpretation of MIC results quite challenging, because the *in vitro* reaction does not necessarily represent the clinical response to treatment (Hadrich & Ayadi 2018).

In this study, we showed the capacity of biofilm production by filamentous fungal isolates. Other similar studies have also described *in vitro* biofilm formations by species of the order Mucorales (Singh *et al.* 2011) and the genera *Scedosporium* (Rollin-Pinheiro *et al.* 2017) and *Trichosporon* (Montoya *et al.* 2018). According to our results, Thomaz *et al.* (2018) reported *Candida* isolates with low biofilm production capacity. However, Pires *et al.* (2011), evaluating yeasts from the hydraulic circuit of a hemodialysis unit, found a considerable number of highly producing strains of the *C. parapsilosis* complex. Similarly, this characteristic has also been demonstrated in other yeasts (Silva *et al.* 2011; Pannanusorn *et al.* 2013).

Commonly, the different techniques for *in vitro* evaluation of the biofilm production capacity of fungal isolates are adapted from methods applied in bacteriological investigations, so they still need to be standardized (Pires *et al.* 2011; Montoya *et al.* 2018). Thus, in addition to the origin of the species, differences in experimental conditions (such as pH, culture medium, oxygen availability, among others) may be responsible for different findings in the literature (Pannanusorn *et al.* 2013). In this study, all parameters were standardized with adequate training for satisfactory fungal growth.

Furthermore, we observed in this study a positive correlation between the MIC values for some antifungals and the absorbance values of the biofilm production test. Likewise, greater antifungal resistance to amphotericin B and fluconazole has been associated with greater capacity for biofilm production by *Trichosporon asahii* isolates in research conducted by Sun *et al.* (2012) and Montoya *et al.* (2018). However, another study did not find this possible correlation (Iturrieta-González *et al.* 2014), suggesting that such results can be influenced by multiple factors, including the origin of the isolate, fungal species, investigated drug, methodological parameters, and others (Ramage *et al.* 2012). Therefore, further studies are needed to elucidate these findings, once the treatment/elimination of biofilm is a major clinical and environmental challenge.

Undoubtedly, the production of biofilm can facilitate the proliferation of microorganisms in different points of the water treatment and distribution systems of the hemodialysis units, in addition to providing greater resistance to cleaning and disinfection procedures, because biofilms are less susceptible to antimicrobial agents compared to planktonic cells (Kanamori *et al.* 2016; Montoya *et al.* 2018).

CONCLUSIONS

This study showed a wide variety of fungi in dialysis water and dialysate samples from all the investigated hemodialysis units. Considering the pathogenic potential of the isolated fungi, the occurrence of these microorganisms in these samples may represent a risk to the health of patients undergoing hemodialysis, who commonly have a compromised immune system and other comorbidities, which make them more susceptible to opportunistic infections. Although more studies are needed to elucidate the clinical potential and the threshold of fungal contamination in hemodialysis fluids, aiming to increase the safety of the dialysis process, our findings suggest the adoption of control measures and regular monitoring of fungi in dialysis water and dialysate.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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