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Black Yeasts-Like Fungi Isolated from Dialysis Water in Hemodialysis Units

Izabel Cristina Figel · Paulo Roberto Dantas Marangoni · Sabina Moser Tralamazza · Vânia Aparecida Vicente · Patrícia do Rocio Dalzoto · Mariana Machado Fidelis do Nascimento · G. Sybren de Hoog · Ida Chapaval Pimentel

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Abstract Hemodialysis in patients with chronic renal failure promotes the removal of toxic substances, water, and minerals from the body and often takes place in specialized clinics. Microbial contamination of dialysis fluid is a serious problem in therapy. One of the sources of contamination is the water used to prepare the dialysate. In Brazil, legislation regulating the microbiological quality of water for dialysis does not cover waterborne microbes such as *Pseudomonas*, mycobacteria, and fungi. The aim of the present study was to quantify, isolate, and identify fungi present in water systems in six hemodialysis units in Curitiba, Paraná state, Brazil. Fungi were analyzed by surface plating and membrane filtration. Isolates were identified by morphology, while the dematiaceous fungi were identified by sequencing the rDNA ITS region. It was found that 66 % of the samples presented fungi, while black fungi were present in 46 % of all samples. Twenty-eight isolates from treated water for dialysis and dialysate were identified by sequencing and were found to be *Exophiala pisciphila*, *E. cancerae*, *E. equina*, and *Rhinocladiella similis*. The presence of dematiaceous fungi may pose a risk for debilitated hospitalized patients.

Keywords Dematiaceous fungi · Dialysis · *Exophiala* · Black yeasts

Introduction

Patients with chronic renal insufficiency are often submitted to procedures such as peritoneal dialysis

I. C. Figel · P. R. D. Marangoni · S. M. Tralamazza · V. A. Vicente · P. do Rocio Dalzoto · M. M. F. do Nascimento · G. S. de Hoog · I. C. Pimentel (✉)
Microbiology, Parasitology and Pathology Post-Graduation Program, Basic Pathology Department, Federal University of Paraná State, Curitiba, Brazil
e-mail: ida@ufpr.br

I. C. Figel
Laboratory of Microbiology and Toxicology, Institute of Technology of Paraná (TECPAR), Curitiba, Brazil

G. S. de Hoog
CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands

G. S. de Hoog
Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Amsterdam, The Netherlands

G. S. de Hoog
Peking University Health Science Center, Beijing, China

G. S. de Hoog
Sun Yat-sen University, Guangzhou, China

G. S. de Hoog
Second Medical Military University, Shanghai, China

and hemodialysis, in which large amounts of water are employed for the production of dialysis fluid [1, 2]. The chemical and microbiological quality of this water must be assured by treatment processes, such as reverse osmosis, in order to avoid transfer of toxic or microbiological contaminants to the patients [3].

The Brazilian Health Ministry (Resolution 154/2004) [4] and the Association for the Advancement of Medical Instrumentation [5] have suggested standards for the certification of water quality in those systems. It is well established that the water treated for dialysis must be total coliform-free, heterotrophic bacteria should not exceed 200 CFU/mL, and the limit concentration of bacterial endotoxin is 2 UE/mL. For the dialysate must be total coliform-free and the number of heterotrophic bacteria should not exceed 2,000 CFU/mL [2]. These requirements are limited in that other microorganisms and metabolites are not investigated, such as *Pseudomonas* species, mycobacteria, fungi, and mycotoxins [6, 7].

Literature data on fungal presence in dialysis water are scant. Clark et al. [8] reported a bloodstream infection with *Phialemonium* sp. from hemodialysis patients and Rao et al. [9] analyzed cases of infection with *Phialemonium curvatum*, concluding that the source of contamination was probably the dialysis water. Arvanitidou et al. [10] described the occurrence of filamentous fungi and yeasts in 85 hemodialysis units in Greece and found that *Aspergillus* was the most prevalent and *Penicillium* the most frequent in tap water and treated water for dialysis and dialysate. The authors also encountered other filamentous fungi, such as *Verticillium*, *Acremonium*, *Alternaria*, *Curvularia*, *Cladosporium*, in addition to *Candida*. Porteous et al. [11] isolated a black yeast from treated dental unit waterlines, identified as *Exophiala mesophila*. The fungal flora in groundwater-derived public drinking water in North Rhine-Westphalia, Germany, contained *Acremonium*, *Exophiala*, *Penicillium,* and *Cadophora* species [12].

Several groups of waterborne fungi may be involved in human mycoses. The order Chaetothyriales contains melanized fungi and black yeasts that show relatively frequent opportunism in healthy patients [13], particularly in the anamorphic genera *Exophiala*, *Cladophialophora*, *Phialophora*, and *Ramichloridium* [14, 15]. *Exophiala* species are known as etiologic agents of superficial, subcutaneous, and systemic infections. Among the most serious pathogens are *E. dermatitidis* [16] and *E. asiatica* [17] which grow at high temperatures (37–45 °C), which is considered a main virulence factor [15]. Similar thermotolerance is observed in hydrophobic, neurotropic genus *Cladophialophora* [18].

During preliminary tests conducted at the Institute of Technology of Paraná, Curitiba, Brazil, the presence of fungal contamination in water of dialysis systems was verified, with accent on black yeast-like fungi. Their presence is of concern. These fungi have a waterborne lifestyle and were detected in treated water for dialysis as well as in the dialysate. There are no requirements for fungal control in the legislation of dialysis water. Research into the amount and frequency of fungi in these systems is deemed necessary in order to establish control parameters. This investigation may lead to an improvement of the standard parameters which could be adopted to monitor dialysis systems.

Materials and Methods

Sampling

The study was approved by the Research Ethics Committees of Federal University of Paraná State (CEP/SD: 799.134.09.09 CAAE: 0059.0.091.091-09) and was conducted over a period of 12 months in six hemodialysis units (A, B, C, D, E and F Clinics) in Curitiba, Paraná state, Brazil, from October 2009 to November 2010. At the end of the study, 217 samples had been analyzed. The collection points were (1) water inlet from the municipal supply; (2) treated water for dialysis from faucets of the reuse room; (3) dialysate collected at the outlets of the dialysis machine; and (4) treated water for dialysis before distribution (Fig. 1). Samples were analyzed in the Laboratory of Microbiology and Toxicology of the Institute of Technology of Paraná—TECPAR and LABMICRO/UFPR.

Fungal Isolation

Viable cells counts were performed using membrane filters (Millipore) as prescribed by the American Public Health Association [19]. A volume of 100 mL was filtered through membrane filters of 47 mm in diameter with micropores of 0.45 μm. The membranes were transferred to dextrose potato agar (PDA) pH 3.5 Acumedia supplemented with tartaric acid 10 % and incubated at 25 ± 1 °C for 15 days, being checked

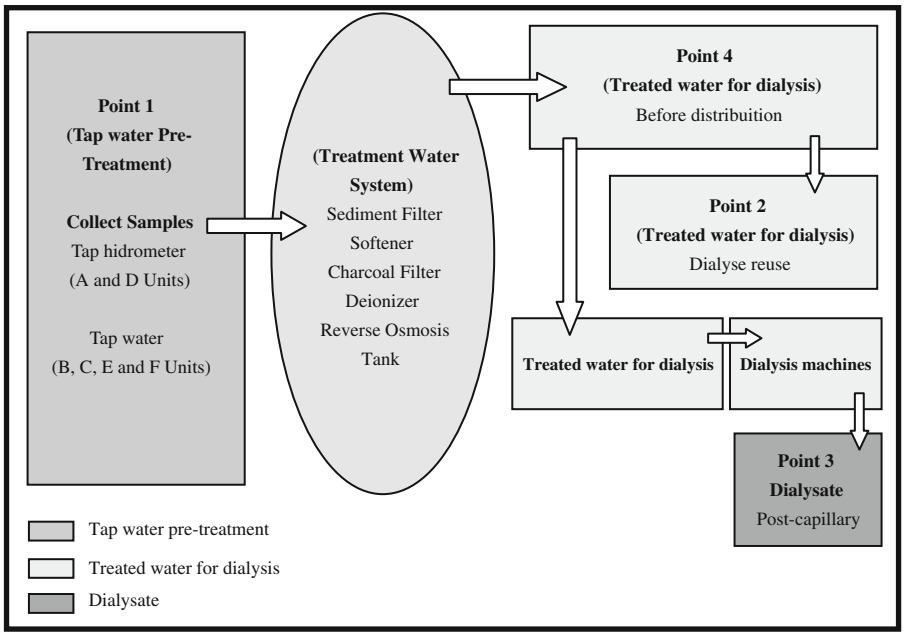

Fig. 1 Points collected in clinical hemodialysis of samples of tap water, treated water for dialysis and dialysate

every day, for assessment of fungi with slow growth. Colonies were counted and the results were expressed as the mean of colony-forming unit (CFU) per 100 mL [19]. Positive cultures of filamentous fungi were cultivated on Sabouraud's dextrose agar. Single-spore colonies were selected for identification. The assays were performed in duplicate.

Identification of Filamentous Fungi

Preliminary identification was carried out using macro- and microscopic features of the colonies after slide culturing on Sabouraud's dextrose agar at 25 ± 1 °C [20]. Melanized fungi were primarily identified as described above and samples with positive identification for the genera *Exophiala*, *Cladosporium*/*Cladophialophora*, and *Rhinocladiella* were also identified by molecular markers.

Molecular Identification

DNA extraction: Approximately 1 cm^2 of 14–21-day-old cultures was transferred to a 2 mL Eppendorf tube containing 400 µL TEx buffer (pH 9.0) and glass beads (Sigma G9143). The fungal material was homogenized with a MoBio vortex for 1 min. Subsequently, 120 µL SDS 10 % and 10 µL proteinase K were added and incubated for 30 min at 55 °C, and the mixture was vortexed for 3 min. After addition of 120 µL of 5 M NaCl and 1/10 vol CTAB 10 % (cetyltrimethylammonium bromide) buffer, the material was incubated for 60 min at 55 °C. Then, the mixture was vortexed for 3 min. Subsequently, 700 µL chloroform:isoamylalcohol (24:1) was mixed carefully by hand and centrifuged for 5 min at 4 °C at 20,400*g* force value. The supernatant was transferred to a new Eppendorf tube with 225 µL 5 M NH_4-acetate, mixed carefully by inverting, incubated for 30 min on ice water, and centrifuged again for 5 min at 4 °C at 20,400*g* force value. The supernatant was then transferred to another Eppendorf tube with 0.55 vol isopropanol and centrifuged for 5 min at 20,400 *g* force value. Finally, the pellet was washed with 1,000 µL ice-cold 70 % ethanol. After drying at room temperature, it was resuspended in 100 µL TE buffer (Tris 0.12 % w/v, Na-EDTA 0.04 % w/v) [14].

Amplification and Alignment

Fragments of rDNA Internal Transcribed Spacer (ITS) were amplified using primers V9G (5′TTACGTCC CTGCCCTTTGTA3′) and LS266 (5′GCATTCCCAA ACAACTCGACTC3′) [21] and sequenced with ITS1 (5′TCCGTAGGTGAACCTGCGG3′) and ITS4 (5′TCC TCCGCTTATTGATATGC3′) [22]. PCR reaction was

performed in 10 μL volumes of a reaction mixture containing sterile distilled water, 0.5 μL PCR buffer (10×, Applied Biosystems), 1 μL of primer (50 pmol), 0.5 μL of Big Dye (Applied Biosystems), and 1 μL PCR products. Thirty-five cycles were performed: 96 °C for 10 s (denaturation), 50 °C for 5 s (annealing), 60 °C for 4 min (extension), and 60 s initial and terminal delay. Sequencing was performed on an ABI 3130 automatic sequencer (Applied Biosystems). Sequences were edited using STADEN Package [23]. ITS sequences were aligned on the basis of similarity by means of the sequence editor MEGA 5 [24]. Sequence analysis was performed using the sequence alignment software BLASTn in GenBank and run against a research database at CBS [25]; identified sequences were aligned using CLUSTAL-W 1.7 [26].

Results

A total of 217 samples of water from hemodialyses clinics (tap water, treated water for dialysis, dialysate) were analyzed, of which 66 % (145/217) presented fungi and in 46 % (100/217) showed dematiaceous fungi (Table 1). In 35 % (75/217) of the water samples, more than one morphotype of fungi was presented. In 19 % of the total number of samples investigated, the count of fungal cells exceeded 100 CFU/mL as established by the membrane filtration method. We selected 239 isolates by macro- and micromorphology, of which 89 were derived from tap water, 92 from treated water for dialysis, and 58 from the dialysate collected at the outlets of dialysis machines (Fig. 1). Of these 239 isolates, 123 were identified as black yeast-like fungi by morphology (Table 1).

A preliminary identification of black fungi based on macro- and micromorphology attributed 32 isolates to the genus *Cladophialophora*, 79 to *Exophiala,* and 12 to *Rhinocladiella*. From these 123 isolates, 41 black yeast-like isolates were selected and processed for molecular identification using ITS sequencing. Using these methods, we identified 30 isolates which proved to belong to the fungal order Chaetothyriales comprising the black yeasts and their relatives (Table 2: 3 were identified as *Exophiala* sp., 23 as *E. pisciphila*, 1 as *E. equina*, 1 as *E. cancerae*, and 2 as *Rhinocladiella similis*). One *Cladophialophora*-like strain was sequenced for which we did not find a match in GenBank or in the CBS black yeast database. The majority of strains we morphologically attributed to *Cladophialophora*, however, appeared to be *Cladosporium* species. Four *Exophiala*-like strains were reidentified as *Cadophora fastigiata*, a member of the order Helotiales. For this comparison, positive identification was considered to be achieved if ITS sequence deviation was <1 %.

Discussion

This study of treated water and dialysate of water systems from hemodialysis units in Curitiba, Brazil, conducted over a period of 12 months, confirmed fungal presence in 66 % of the investigated samples. A high prevalence of black fungi was observed, with 46 % of the investigated samples. The abundance of fungal cells was high, as in 19 % of the total number of samples investigated the count of fungal cells exceeded 100 CFU/100 mL when determined by the membrane filtration method (Table 1). Although there is significant fungal contamination in dialysis water systems, national and international standards do not include fungal counts as a parameter to assess the quality of dialysis water. In Sweden, as one of the very few countries with a regulation that involves fungi, the upper limit for microbial water contamination is 100 CFU/mL considering the total number of microorganisms, and fungi should be <10 CFU/mL [1].

Varo et al. [27] studied water samples in a hemodialysis unit in São Paulo State in Brazil and observed filamentous fungi in 100 % of the investigated samples. Pires-Gonçalves et al. [7] also found dematiaceous fungi in water from a hemodialysis center in São Paulo State, which were identified as species of *Aureobasidium*, *Cladosporium*, *Exophiala*, and *Curvularia*. In the present study, black fungi were found in the water inlet of the municipal supply and in treated water for dialysis and dialysate in five dialysis clinics, indicating that the fungi were not eliminated during water treatment processes. Molecular studies confirmed the presence of three *Exophiala* species, viz. *Exophiala pisciphila*, *E. cancerae*, and *E. equina*. In addition, in one clinic (F), black fungi were found in treated water for dialysis only, with two isolates identified as *R. similis* (Table 2).

The potential health risk of fungal contamination in dialysis water for immunocompromised patients has to

Table 1 Presence of fungi including black yeasts in tap water, treated water for dialysis and dialysate

Points collected	Samples with fungi	Samples above cut-off[a] value for fungi	Sample with presence of fungi Black yeast	Samples with threshold value[a] exceeded for black yeasts,
All points	66 % (145/217)	19 % (43/217)	46 % (100/217)	12 % (28/217)
Point 1 (tap water)	50	19	38	19
Point 2 (treated water for dialysis)	52	12	36	7
Point 3 (dialysate)	37	10	21	1
Point 4 (treated water for dialysis)	6	2	5	1

[a] Threshold concentration proposed in this study: above 100 CFU/100 mL by membrane filtration

be established. The black yeast-like fungi seem to be an adequate fungal parameter for the microbiological quality of water, because of their rather consistent presence [7, 8, 10, 15] and because of their potential ability to cause disease in vertebrates with or without apparent underlying disease [14, 16]. Melanized fungi are common saprobes in indoor environments [14], where they seem to occupy specific microhabitats. This is probably stimulated by a low competitive ability toward co-occurring microorganisms, as is judged from the fact that their isolation is enhanced by the use of semi-selective methods [14]. Their oligotrophism enables them to thrive and maintain at low density on adverse substrates where common saprobes are absent [28]. Drinking water systems provide such habitats.

Despite the low frequency in clinical practice, the melanized fungi of the Chaetothyriales have become increasingly recognized as important pathogens because of the severity of infections [29]. Individuals with an apparently intact immune system have been reported to have invasive, often fatal infections [16, 30]. It is expected that immunocompromised or otherwise debilitated patients are at increased risk. Nucci et al. [31] related a nosocomial outbreak of *Exophiala jeanselmei* fungemia to contamination of hospital water. Environmental cultures yielded *E. jeanselmei* from 3 of 85 sources investigated, including deionized water from the hospital pharmacy, a water tank, and water from a sink in a non-patient care area. Molecular typing results showed that isolates from symptomatic patients and isolates from pharmacy water were identical, whereas the patterns of isolates recovered from two other sources of water were distinct. Several authors have stressed that screening black fungi in indoor environments enhances our understanding of eventual health risks [32–34]. A reliable taxonomic system is mandatory to obtain insight in the link between clinical disease and environmental ecology [14].

The morphological identification adopted in this study allowed identification of isolates down to the genus level, while ITS sequencing provided species identities. With the CBS data base as a Ref. [25], strains 78/1011-09F2 and 162/143-F2 isolated from treated water for dialysis (Fig. 1) were identified as *R. similis* (Table 2). This species was previously isolated from a biofilter eliminating gasoline vapors [35] and is also known from human skin [36].

Among the isolates of *Exophiala*, the species encountered were known waterborne species: *E. pisciphila*, *E. equina*, and *E. cancerae* [15]. *E. pisciphila* was reported from a liver transplant recipient presenting skin papules, which eventually drained [37]. *Exophiala equina* originated from different kinds of cold water and fluids including the cooling system of a packaging machine, the tubing of an instrument using silica gel, and from washings of *Tilia* roots [15]. De Hoog et al. [15] also reported isolates from bathrooms and from bottled spring water for human consumption; one isolated was derived from dialysis tubing. Although *E. equina* is unable to grow at 37 °C, some superficial infections in humans were noted, particularly from skin of the extremities, as well as from stool and sputum. Lian and de Hoog [33] hypothesized a possible transmission route of black yeasts from bathing facilities. The occurrence of *Exophiala* in bottled and dialysis water is of concern, but systemic infections are not expected.

Exophiala cancerae has been isolated from tissue of moribund mangrove crabs (*Ucides cordatus*) as one of the causes of Lethargic Crab Disease (LCD), a systemic disease which has caused extensive epizootic mortality of crabs along the Brazilian coast [38]. Additional strains of *E. cancerae* were isolated on

Table 2 Fungi isolated from water systems in six dialysis clinics in Curitiba, Brazil

Isolate number	Source/sampling area	Gene sequenced/GenBank number	Identity	% Identity
88/46-B2	Dialysis water/Clinic B	ITS/JX839449	*C. fastigiata*	100
120/46-B2	Dialysis water/Clinic B	ITS/JX839450	*C. fastigiata*	100
176/229-B2	Dialysis water/Clinic B	ITS/JX839451	*C. fastigiata*	100
177/229-B2	Dialysis water/Clinic B	ITS/JX839452	*C. fastigiata*	100
03/830-09A3	Dialysate/Clinic A	ITS/JN650527	No match	
128/949-A3	Dialysate/Clinic A	ITS/KC354798	*Cladosporium* sp.	100
115/1005-09D2	Dialysis water/Clinic D	ITS/JN650537	*Cladosporium sp.*	99
181/235-D2	Dialysis water/Clinic D	ITS/KC354800	*Cladosporium sp.*	97
182/235-D2	Dialysis water/Clinic D	ITS/JX839453	*Cladosporium sp.*	100
26/845-E2	Dialysis water/Clinic E	ITS/KC354791	*Exophiala* sp.	99
66/996-A2	Dialysis water/Clinic A	ITS/KC354794	*Exophiala* sp.	95
127/845-E2	Dialysis water/Clinic E	ITS/KC354797	*Exophiala* sp.	99
09/833-09B3	Dialysate/Clinic B	ITS/JN650528	*E. pisciphila*	99
13/842-D2	Dialysis water/Clinic D	ITS/KC354789	*E. pisciphila*	99
15/845-E2	Dialysis water/Clinic E	ITS/JX839454	*E. pisciphila*	100
16/846-E3	Dialysate/Clinic E	ITS/KC354790	*E. pisciphila*	99
20/832-09B2	Dialysis water/Clinic B	ITS/JN650529	*E. pisciphila*	99
29/846-E3	Dialysate/Clinic E	ITS/KC354792	*E. pisciphila*	99
34/951-B2	Dialysis water/Clinic B	ITS/KC354793	*E. pisciphila*	99
40/952-09B3	Dialysate/Clinic B	ITS/JN650530	*E. pisciphila*	99
45/960-09E2	Dialysis water/Clinic E	ITS/JN650531	*E. pisciphila*	99
53/960-09E2	Dialysis water/Clinic E	ITS/JN650532	*E. pisciphila*	100
56/999-B2	Dialysis water/Clinic B	ITS/JX839455	*E. pisciphila*	100
69/1000-B3	Dialysate/Clinic B	ITS/JX839456	*E. pisciphila*	100
75/1008-E2	Dialysis water/Clinic E	ITS/JX839457	*E. pisciphila*	100
76/1009-E3	Dialysate/Clinic E	ITS/JX839458	*E. pisciphila*	100
89/47-B3	Dialysate/Clinic E	ITS/JX839459	*E. pisciphila*	100
99/56-E3	Dialysate/Clinic E	ITS/JX839460	*E. pisciphila*	100
109/999-B2	Dialysis water/Clinic B	ITS/KC354796	*E. pisciphila*	100
144/132-B3	Dialysate/Clinic B	ITS/JX839461	*E. pisciphila*	100
150/135-10C3	Dialysate/Clinic C	ITS/JN650533	*E. pisciphila*	99
160/137-10D2	Dialysis water/Clinic D	ITS/JN650534	*E. pisciphila*	98
178/230-B3	Dialysate/Clinic C	ITS/JX839462	*E. pisciphila*	100
189/239-10E3	Dialysate/Clinic E	ITS/JN650536	*E. pisciphila*	99
141/131-B2	Dialysate/Clinic B	ITS/KC354799	*Exophiala pisciphilla*	100
188/238-E2	Dialysis water/Clinic E	ITS/JX839463	*E. cancerae*	100
168/226-10A2	Dialysis water/Clinic A	ITS/JN650535	*E. equina*	100
2/829-A2	Dialysis water/Clinic A	ITS/KC354788	*M. laricina*	97
90/47B3	Dialysate/Clinic B	ITS/KC354795	*P. dimorphosporum*	99
78/1011-09F2	Dialysis water/Clinic F	ITS/JN650538	*R. similis*	99
162/143-F2	Dialysis water/Clinic F	ITS/JX839464	*R. similis*	100

separate occasions from water in Germany and The Netherlands [15]. In the same study, skin and nail infections were particularly in patients with diabetes.

In our study, we also encountered strictly saprobic isolates, such as the common contaminants of the genus *Cladosporium* (Table 2). Some black fungi

were identified as *C. fastigiata* anamorphs of *Pyrenopeziza* species in the order Helotiales [15]. This species is known to be common in municipal drinking water [12]. According to de Hoog et al. [15] in this order another ecological trend is observed, featuring opportunism and pathogenicity to plants rather than to animals. We also found one isolate of *Mycosphaerella laricina*, the genus *Mycosphaerella* being a teleomorph of *Cladosporium* (20). Another identified fungus was *Phialemonium dimorphosporum*, Rao et al. [9] isolated *P. curvatum* in two patients with fungemia originating from tap water and from the machine with which the patients underwent hemodialysis.

In the hospital, the incidence of fungal infections has increased in recent years due to several factors, including growing use of antibiotics, immunosuppressive drugs, invasive medical procedures, and because of improved patient survival. In hemodialysis patients, fungal infection is usually associated with vascular access [39, 40]. Infections caused by dematiaceous fungi are uncommon, but have become increasingly recognized because of the wide variety of clinical syndromes [41]. Nucci et al. [31] diagnosed 19 cases of fungemia due to *E. jeanselmei*. Zarei Mahmoudabadi et al. [42] noted contamination by fungi in 4 % of dialysis filters. The presence of fungi in water systems for dialysis is now well established in the literature [7, 8]. Fungal contamination in hemodialysis patients is rarely determined, but the frequency appears to be high upon investigation [8, 27]. Kauffmann-Lacroix et al. [43] concluded that the absence of knowledge about the contamination threshold of nosocomial invasive fungal infection makes it difficult to impose routine monitoring. Since most of the fungi in water are not thermotolerant, the risk of systemic infection is low in all types of patients, but superficial and opportunistic infections by black yeasts and related fungi do occur regularly. Introduction of a threshold concentration of black fungi seems appropriate.

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