

Microbiological quality of water and dialysate from haemodialysis units in Southern Brazil

Qualidade microbiológica da água e dialisato em clínicas de hemodiálise do Sul do Brasil

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ABSTRACT

Infectious diseases in renal patients may be associated with the dialysis water quality, which may be contaminated with microorganisms. In Brazil, the water quality is evaluated by analyzing total coliforms, heterotrophic bacteria, and bacterial endotoxin, but not *Pseudomonas* sp. and fungi. Water samples from haemodialysis units in Curitiba/PR were investigated on their conformity with the standard established by the Brazilian Health Ministry. Total coliforms, heterotrophic bacteria, *P. aeruginosa* and fungi counts were performed according to APHA, and LAL methodology for detecting bacterial endotoxin. All of the samples showed the total coliforms counts ≤ 1.1 MPN/100 mL, and ≥ 95 % of analyzed samples complied with the standards for heterotrophic bacteria counting. *P. aeruginosa* was recovered from 4 % of samples. In 15 % of samples, bacterial endotoxin was detected in values above the limit established by legislation. Yeasts were isolated from 26 % samples and filamentous fungi from 58 %, being 46 % characterized as melanized fungi. The fungi genera were *Cladosporium* spp., *Penicillium* spp., *Beauveria* spp., *Exophiala* spp., *Fusarium* spp., *Aspergillus* spp., *Trichoderma* spp., *Acremonium* spp. and *Rinocladiella* spp.. The study highlights the significance of *P. aeruginosa* and fungi detection in those systems, as these microorganisms are potentially pathogenic to immunocompromised patients.

Keywords. *Pseudomonas aeruginosa*, coliforms, heterotrophic bacteria, endotoxins, fungi.

RESUMO

Doenças infecciosas em pacientes renais podem ser associadas à qualidade da água de diálise, que pode apresentar contaminação com microrganismos. Pelos padrões brasileiros, a qualidade da água é avaliada analisando-se coliformes totais, bactérias heterotróficas e endotoxinas bacterianas. *Pseudomonas* sp. e fungos não são investigados. Amostras de água de clínicas de hemodiálise em Curitiba/PR foram avaliadas quanto à conformidade com os padrões do Ministério da Saúde. Contagens de coliformes totais, bactérias heterotróficas, *Pseudomonas aeruginosa* e fungos foram realizadas seguindo-se APHA e detecção de endotoxina pela metodologia LAL. Todas as amostras tiveram contagens de coliformes totais abaixo de 1,1 MPN/100 mL e ≥ 95 % das amostras apresentaram padrões aceitáveis para bactérias heterotróficas. *P. aeruginosa* foi encontrada em quatro amostras. Em 15 % das amostras, endotoxinas bacterianas foram detectadas em valores acima dos permitidos pela legislação. Leveduras foram isoladas em 26 % das amostras e fungos filamentosos em 58 %, sendo 46 % melanizados e 27 % hialinos. Os gêneros fúngicos detectados foram *Cladosporium* spp., *Penicillium* spp., *Beauveria* spp., *Exophiala* spp., *Fusarium* spp., *Aspergillus* spp., *Trichoderma* spp., *Acremonium* spp. e *Rinocladiella* spp.. Foi evidenciada a importância da detecção de *P. aeruginosa* e fungos nestes sistemas, uma vez que estes podem ser potencialmente patogênicos para pacientes imunocomprometidos.

Palavras-chave. *Pseudomonas aeruginosa*, coliformes, bactérias heterotróficas, endotoxinas, fungos.

INTRODUCTION

The Brazilian Society of Nephrology estimates that about 10 million people are affected by different levels of Chronic Renal Disease in Brazil¹. About 89.4 % of these patients had been submitted to hemodialysis and 10.6 % to peritoneal dialysis. The mortality observed was 15.2 % per year mostly due to the cardiovascular (37 %) and brainvascular (10 %) complications, infectious (26 %) and others not identified (27 %)².

Infectious diseases in renal patients can be associated to the dialysis water quality, in which several microorganisms, such as bacteria, viruses, fungi, protozoa, algae and also bacterial toxins can be assessed, mostly due to the inefficacy in the water treatment process³. Figel et al⁴ detected the presence of fungi in 66 % of water samples assayed from dialysis units in Southern Brazil, and observed the high prevalence (46 %) of black fungi, that can be associated to several diseases related to immunocompromised patients. The bacterial cytokine-inducing substances, as lipopolysaccharides (endotoxins), exotoxins and peptidoglycans, are also a concern, as they can

be transferred to the patient blood compartment during dialysis. The exposure to high levels of endotoxins is obviously associated with pyrogenic reactions and septicemia⁵. In Brazil, standards had been established for the certification of water quality in those systems⁶. The microbial standards for water for dialysis includes absence of coliforms, heterotrophic bacteria count limits of 200 CFU/mL and endotoxin limits of is 2 EU/mL; and for dialysate, heterotrophic bacteria count limit, the only parameter predicted for, of 2000 CFU/mL⁷. Although those parameters are important and necessary, others microorganisms and metabolites should be investigated in dialysis water systems, such as *Pseudomonas* sp., mycobacteria, fungi and mycotoxins⁸.

We aimed the evaluation of the microbial quality of water for dialysis provided by Hemodialysis Units in Curitiba, Paraná, Brazil, in attempt to verify the accomplishment of the standards demanded by Brazilian Health Ministry. In addition, counts of *Pseudomonas aeruginosa* and fungi were also performed in attempt to highlight its importance and propose its inclusion in future revisions of the standards for water for dialysis.

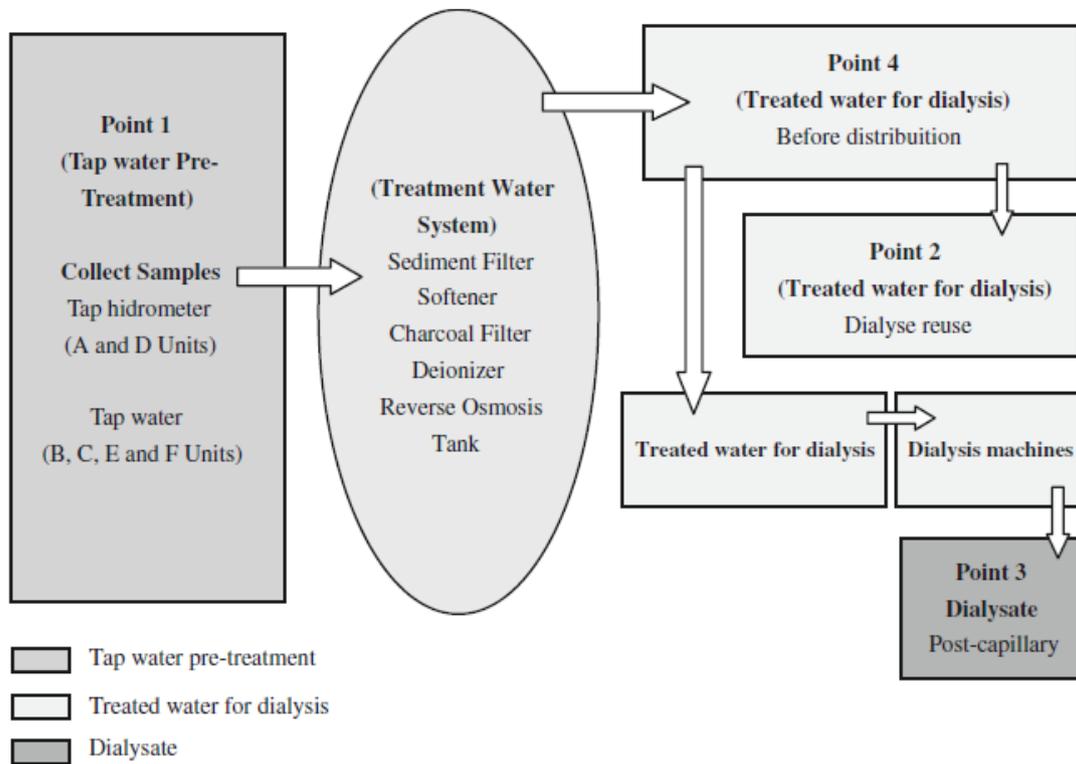


Figure 1. Points collected in clinical hemodialysis of samples of tap water, treated water for dialysis and dialysate (Figel et al, 2013)

MATERIAL AND METHODS

Water sampling

Samples of tap water (point 1), treated water for dialysis (point 2) and dialysate into the dialysis system (point 3) were obtained from six Hemodialysis Units in Curitiba, PR, Brazil (Figure 1).

The collections were performed monthly from October 2009 to November 2010. The sampling points were previously disinfected by applying 70 % alcohol to faucet and 400 mL of water flowed freely after the treatment, to prevent sample contamination with the disinfectant utilized. The samples were collected to sterile 250 mL glass flasks after 30 seconds of free flow. To the flask for the point 1 had been added 10 % sodium thiosulfate and the flasks for bacterial endotoxin analysis were previously depyrogenated.

The microbiological analysis of the samples (217) were performed within 12-h after collection, at the Laboratory of Microbiology and Toxicology of the Paraná Institute of Technology – TECPAR and LabMicro/UFPR.

The research was approved by the Committee of Ethics in Research of Federal University of Parana (CEP/SD: 799.134.09.09 CAAE: 0059.0.091.091-09).

Methods

Regarding the standard parameters for dialysis water in Brazil⁶, we performed the heterotrophic bacteria count test by pour plate method, total coliforms count test by multiple-tube method in 100 mL⁹, and detection of bacterial endotoxin by the *Limulus* amoebocyte lysate (LAL) method¹⁰. Although not necessary according to Brazilian standards, other parameters were also evaluated: *P. aeruginosa* count test by multiple-tube method and fungi count by spread plate and membrane filter method⁹.

Heterotrophic bacteria count

10 mL of the sample was added to a diluent to make a 1:10 dilution. 1-mL aliquots of the sample and the 1:10 dilution preparation were separately added to Petri dishes. The molten PCA medium (0.5 % peptone, 0.25 % yeast extract, 0.1 % glucose, 1.5 % agar; pH 7.0) was added to each

plate, which was incubated at 35 °C ± 1 °C for 48 h. At the end of the incubation period, the recovered colonies from each plate were enumerated and the results were expressed in colonies forming units (CFU) per mL⁹. The experiments were conducted in duplicate.

Total coliforms

The total coliforms counts were assessed by the most probable number (MPN) method⁹. 10-mL aliquots of the samples were added to a set of 10 tubes, each containing double-strength Lauryl Sulphate broth added of blue bromocresol (Hymedia) and an inverted Durham tube. All tubes were incubated at 35 °C ± 1 °C for 48 h. Following incubation, the tubes that were media acidification was observed and/or gas was noticed inside the Durham tubes were submitted to confirmation test for total coliform. Each presumptively positive tube was subcultured into a Brilliant Green Bile 2 % broth (Hymedia) and incubated at 35 °C ± 1 °C for 48 h. Positive results indicate the presence of total coliforms and the count was expressed as MPN/100 mL⁹.

Endotoxins

The bacterial endotoxin test was performed using the *Limulus* amoebocyte lysate (LAL) by gel-clot limit test. Each sample was diluted using water for Bacterial Endotoxins Test until the maximum valid dilution (MVD), that was calculated from equation $MVD = \text{endotoxin limit for water for dialysis} / \lambda$ [where λ is the labeled sensitivity of LAL reagent in the gel-clot technique]. Equal volumes of sample and LAL reagent were transferred to test tubes, mixed and incubated at 37 °C ± 1 °C for 1 h in water bath, in order to verify a firm gel formation at the bottom of the tubes. Positive control for endotoxin production was CSE Standard Endotoxin *E. coli* 055:B5. The results were expressed by endotoxins units (EU)/mL¹⁰.

Pseudomonas aeruginosa

P. aeruginosa count was performed by MPN/100 mL⁹. A 10-mL aliquot of the sample was inoculated into 10 tubes containing double-strength asparagin broth (Hymedia). The inoculated tubes were incubated at 35 °C ± 1 °C for 48 h. Tubes showing turbidity and/or pigmentation were submitted to

confirmation. 0.1 mL of cultures in positive tubes were inoculated into acetamide broth (Himedia) and onto the surface of cetrimide agar, all incubated at 35°C ± 1°C for 48 h. The development of a purple color at acetamide broth and presence of a green fluorescent pigment at cetrimide agar indicated the presence of *P. aeruginosa*. The results were expressed in MPN/mL.

Fungi

Fungi were counted by spread plate method and by membrane filter technique⁹.

Spread plate method

10 mL of the sample was added to a diluent to make a 1:10 dilution. 0.1-mL aliquots of the sample and the 1:10 dilution preparation were separately transferred onto Petri dishes containing Potato Dextrose Agar (Himedia) added of tartaric acid 10 %, pH 3.5, and the samples were spread over the surface of the culture medium using a sterile Drigalski spatel. The plates were incubated at 25 °C ± 1 °C for 5 days (faster growth fungi) and 20 days (slower growth fungi). The experiment was conducted in duplicates.

Membrane filter method

100 mL of sample were filtered through a sterile 47-mm, 0.45-µm-pore-diam membrane filter. The membrane was placed on PDA medium, pH 3.5 (Himedia) supplemented with tartaric acid 10 % in Petri dish, that was incubated at 25 °C ± 1 °C for 15 days. Colonies were enumerated and the results were expressed CFU/100 mL⁹. Positive cultures

of filamentous fungi were inoculated on Sabourad dextrose agar (Himedia), incubated at 25 °C ± 1 °C, and the single-spore colonies were selected for identification, carried out using macro and microscopic features. The assays were performed in duplicates.

RESULTS AND DISCUSSION

More than 95 % of the samples were considered in accordance with the standards required by valid Brazilian legislation in 2009 in regard of heterotrophic bacteria counts. All samples resulted in absence of total coliforms (< 1.1 MPN/100 mL), as predicted by legislation. Abbas et al¹² analyzed total coliforms in 225 samples from hemodialysis units in Greece and observed that 4,9 % of the samples assayed showed counts not acceptable for the established standards.

P. aeruginosa was recovered from 4 % of the samples (8/217) and this result confirm De Filippis et al³ suggestion that *P. aeruginosa* as an additional parameter for assuring the quality of water for dialysis.

15% of the samples showed bacterial endotoxin > 2 EU/mL. However, 32 % of the samples showed values < 0.125 EU/mL, in accordance to the parameters established by Brazilian legislation and standards from Italy, Sweden, Germany and Norway.

Hyaline yeasts were recovered from 26 % of the samples (57/217) and filamentous fungi, from 58 % of the samples (125/217) - 46 % characterized as melanized fungi and 27 % as hyalines. The higher isolation was observed when the membrane filter method was employed (Table 1).

Table 1. Percentage of yeasts and filamentous fungi verified in water for dialysis using spread plate and membrane filter methods

Collecting points	Spread plate	Membrane filter
	Yeasts	
All points	9 % (19/217)	26 % (56/217)
Point 1 (tap water)	1 % (1/72)	12 % (9/72)
Point 2 (dialysis water)	14 % (10/72)	39 % (28/72)
Point 3 (dialyzate)	14 % (8/58)	31 % (18/58)
Point 4 (dialysis water)	0 % (0/15)	7 % (1/15)
	Filamentous fungi	
All points	21 % (46/217)	52 % (112/217)
Point 1 (tap water)	31 % (22/72)	61 % (44/72)
Point 2 (dialysis water)	18 % (13/72)	60 % (43/72)
Point 3 (dialyzate)	16 % (9/58)	36 % (21/58)
Point 4 (dialysis water)	13 % (2/15)	27 % (4/15)

Despite of the fungi contamination observed in the dialysis units, the national and the most of the international legislation do not include these microorganisms as indicators of quality for water for dialysis, except in Sweden, where the current standards for fungi are 10 CFU/mL¹³.

The fungal isolates were identified as *Cladosporium* spp., *Penicillium* spp., *Beauveria* spp., *Exophiala* spp., *Fusarium* spp., *Aspergillus* spp., *Trichoderma* spp., *Acremonium* spp. and *Rinocladiella* spp. Figel et al⁴ isolated black fungi from the water inlet of the municipal supply and in treated water for dialysis and dialysate by five dialysis clinics; three *Exophiala* species were characterized, *Exophiala pisciphila*, *E. cancerae* and *E. equina*. Schiavano et al¹⁴ evaluated 976 water samples and recovered filamentous fungi in 96 samples, yeast isolates in 28, and six samples contained both moulds and yeasts. These authors described 26 genera of fungi, many of which are known as opportunistic pathogens, such as *Cladosporium* spp., *Alternaria* spp. and *Tricophyton* spp. Fungal counts in treated water and standard dialysate solution were always below the threshold (< 10 CFU/mL), in agreement with the Italian guidelines for dialysis fluid quality, however 10.9 % of the samples of ultrapure dialysate solution were contaminated by one or several fungi types.

These results highlight the importance of contaminants detection in dialysis systems, as many infections have been described in immunocompromised patients. The parameters not practiced in Brazil, *P. aeruginosa* and fungi count, and also the detection of melanized fungi, indicate the necessity of further analysis in those systems, in regard to the patients' health.

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