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ORIGINAL ARTICLE

Bacteriological water quality and biofilm formation in the treatment system of the hemodialysis unit in Tlemcen, Algeria

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Abstract

Objective: To evaluate and compare the microbiological quality of osmosis water at the distribution loop, at the dialysis generator inlet and to study the prevalence of biofilm in the tubing.

Methods: Microbiological analysis of 20 water loop samples, 10 water samples were taken at the machine entry and 10 pipe segments from tubing connecting the machines to the loop was done.

Results: The bacterial enumeration results of the loop water vary from 90 to 150 CFU/mL, while the average number of bacteria at the entry of the machines was 182 CFU/mL. The counts of the adhered bacteria in the tubing were worrying with rates ranging from 4.30 to 6.74 Log CFU/ cm^2. Fifty percentage of the strains isolated were *Bacillus*, followed by *Enterobacter cloacae* 23.52%, Staphylococcus, and others such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumanii*. More than half of the tubing strains were highly formative of biofilm, 13 strains with medium capacity and 10 were weakly.

Conclusion: This study indicates bacterial water contamination. The formation of a biofilm will certainly harm the effectiveness of the various disinfection methods in this unit. Water quality is influenced not only by the high rate of bacterial colonization, but also differences in standards for dialysis water.

1 | INTRODUCTION

In recent years, the worldwide number of patients involved in hemodialysis has increased, and this technique is the most commonly used modality for the end-stage renal disease.[1,2]

Patients undergoing dialysis treatment are at a high risk of acquiring systemic infections. Treatment sessions are between 4 and 6 hours where patients are exposed annually and non-selectively to volumes of water varying between 15 000 and 20 000 L of dialysis fluid.[3] Therefore, all substances with low molecular weight in water have direct access to the patient's bloodstream. The failure of water treatment systems, including filtration and reverse osmosis, as well as loop and machines disinfection methods, is responsible for the majority of bacteremia, sepsis, and pyrogenic reactions contracted during hemodialysis.[4-7]

It has been reported that in dialysis water, the greatest incidence is that of Gram-negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumanni* and *Burkholderia cepacia*, Bacillus, and Staphylococcus[8] in addition to some fungi and protozoa, that can multiply rapidly, even in distilled water. In hemodialysis solutions, this bacterial growth may be faster because of the presence of glucose and bicarbonate, which would lead to high concentrations of endotoxins.[9,10] Also, treated water and dialysate samples can be a source of infection by bacteria resistant to various antibiotic agents clinically used.[11]

Bacterial contamination of water in treatment systems and distribution can lead to the formation of biofilms, especially as the treatment station is composed of various types of equipment piping, elbows, loops, muds, and storage tanks, that can be an ideal environment for the biofilms formation. Their presence is worrying because

of bacterial persistence at various points in the system, the continued salting out of bacteria and their components, and the development of greater resistance of sessile bacteria during disinfection procedures,[7] which can be 3000 times more resistant than their planktonic counterparts.

Biofilms can develop on the inner surface of the tubing in the dialysis fluid pathways. This represents a significant risk to the patients' health. This is due to the sliding of the biofilm, which can cause a large but unpredictable release of bacterial and bacterial endotoxins.[12,13]

To reduce the infectious risk of these biofilms, they must be prevented on the surface of dialysis systems, like any medical device because no cleaning and/or disinfection process available has the necessary efficiency.

Today, hemodialysis centers control the contamination of dialysis water and a few of them also control dialysate, but the development of biofilm inside tubing and dialysis machines is not taken into consideration. Besides, the replacement of the tubes is not always done.

In this study, the first in Algeria, our objective was to determine and compare the microbiological quality of the water at the distribution loop and the entry of the dialysis generator, by isolating and identifying Gram-negative and Gram-positive bacteria and studying the prevalence of adhered bacteria from tubing sections that connect the generators to the dialysis loop in a hemodialysis unit of Tlemcen university hospital, Algeria.

2 | METHODS

2.1 | Research site

This study was conducted in a hemodialysis unit of Tlemcen University hospital. The service performs approximately 1000 hemodialysis sessions monthly in three groups per day for end-stage renal disease patients. The water treatment system is an integrated unit in the service that includes the pre-treated water tap with a filtration system, a water softener, and an activated carbon filter followed by a final purification with the water reverse osmosis (RO) treatment process. The treated water is distributed directly to the dialysis machines.

After each hemodialysis session, the machines are rinsed and disinfected according to the protocols applied by the service.

2.2 | Water sampling procedure

Under aseptic conditions, water samples (100 mL) were collected from two sites: osmosis water distribution loop and at the dialysis generator entry.

These two sites are chosen to compare the microbiological quality of water in these two points (Figure 1).

2.3 | Counts

The water samples are put in sterile glass bottles and were carried to the laboratory for immediate analysis. According to the recommendations of the European Pharmacopoeia, the water samples were filtered on cellulose acetate membranes (millipore size 0.45 μm), these are then placed on Petri dishes containing Reasoner's 2A agar (R2A, this minimum medium is recommended to limit the bacterial stress caused by the transfer of bacteria from an oligotrophic medium). In parallel, 0.2 mL of each sample is surface-seeded on R2A agar and nutrient agar. The number of CFU was counted after an incubation period of 5 days at 22°C.

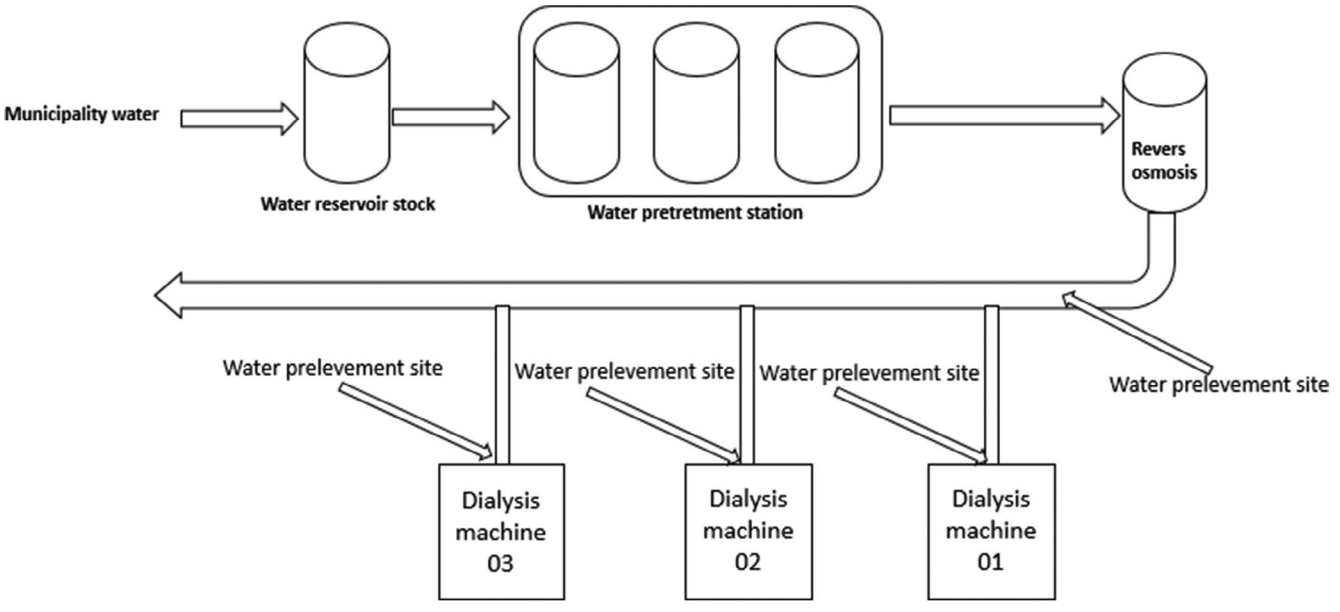

FIGURE 1 Sampling site

2.4 | Identification of isolates

The isolates identification was based on phenotypic characters: Gram, catalase, oxidase, and Api galleries (Bio Mérieux).

2.5 | Treatment of sections of water tubing removed from dialysis machines

Evaluation of the bacteria in biofilm was studied on pre-cut segments of water supply tubing connecting the dialysis generators to the distribution loop, placed in sterile pots and transported aseptically to the laboratory to be analyzed.

The samples were prepared under aseptic conditions, the outer surfaces of the tubings were thoroughly disinfected with alcoholic solution 70%.

The detachment of the bacterial biofilm is carried out according to the technique validated by Smeeth et al., 2003, where they study the detachment of the biofilm in short sections of new and sterile pipes contaminated with known bacterial strains.[14]

In our case, it is cut a 1 cm^2 segment of pipe. A strong swabbing of the internal surface of the segment (pipe) ensures the biofilm detachment. The swabs were immediately put in tubes containing 10 mL of physiological sterile water and then vortexed for 2 minutes to detach the attached bacteria in the swab as much as possible.

After incubation, the colonies were enumerated and the results were expressed in the logarithm of colony-forming units per square centimeter (log CFU/cm^2).

Colonies with morphological differences were isolated and purified. The identification of strains was based on Gram staining and biochemical characters (miniaturized identification system API).

All experiments were performed in duplicate and the averages values were calculated.

2.6 | Study of the biofilm formation capacity of isolated strains

The biofilm formation capacity was tested by the crystal violet microtiter plate method. This technique was carried out according to the recommendation of Christensen and collaborator (1985). The different isolated strains were incubated overnight on the BHIB medium at 37°C.

Each young culture is adjusted to 10^8 CFU/mL and then diluted to 1/100. A volume of 200 µL of this dilution was added to each well. For each strain, three wells were inoculated. The microtiter plate was incubated at a temperature of 37°C for 24 hours.

The wells of the microtiter plates were rinsed three times with sterile distilled water. Then dried and stained with 200 µL of 5% crystal violet for 30 minutes. After the staining step, an abundant wash (five times) was performed with sterile distilled water to remove the weakly adhered cells. The formed biofilm incorporates the crystal violet and to solubilize and measure this amount of coloring, 200 µL of 95% ethanol was added to each well. The optical density measured was 570 nm.

3 | RESULTS AND DISCUSSION

3.1 | Microbiological quality results

For five months, 20 water samples were taken from the distribution loop (3 to 5 samples per month) and another 10 samples were taken just at the entry of each machine.

The count on the R2A medium promotes the growth of bacteria present in the dialysis water and gives more satisfaction than that made on nutrient agar because the number of bacteria counted was greater 106 CFU/mL against 16 CFU/mL respectively.[25] The bacterial enumeration results of the water taken from the distribution loop (Figure 2) varied from 90 to 150 CFU/mL with an average of 118 CFU/mL.

As for the quality of the water at the entry, the various dialysis generators (dialysis machine inlet), this one had a count of bacteria higher than that found in distribution loop water, with an average of 182 ufc/mL; Figure 3). The maximum rates were found at machines M9, M5, and M10 were respectively 220, 202, and 197 CFU/mL.

Since the bacterial count at the entry of the dialysis generators was very important, we wanted to know the contamination and essentially the bacterial adhesion in the water pipes connecting the distribution loop to the different dialysis machines.

The 10 samples of pipe segments corresponding to the 10 dialysis machines show very high contamination by biofilms.

Already the observation with the naked eye of the interior of these segments showed the presence of a thick viscous layer.

The enumeration results of adhered bacteria (biofilm) on 1 cm^2 are worrying with rates ranging from 4.30 Log CFU/cm^2 to 6.74 Log CFU/cm^2 respectively for machines 7 and 9 (Figure 4).

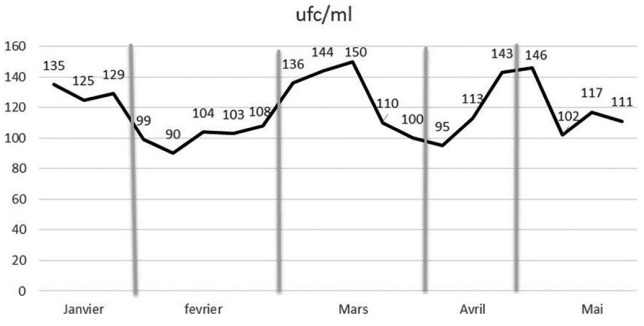

FIGURE 2 Bacterial count of dialysis water taken from the distribution loop for five months

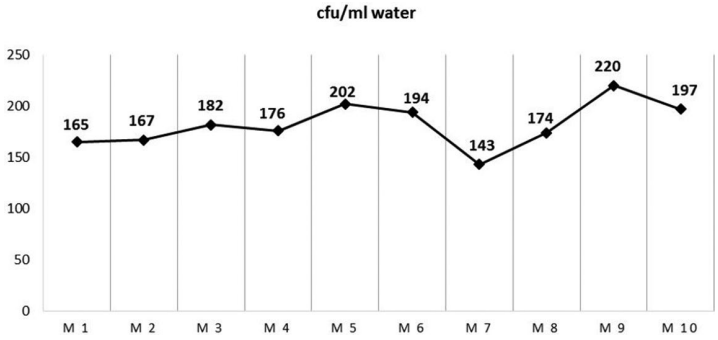

FIGURE 3 Bacterial count of dialysis water taken from the generator entry

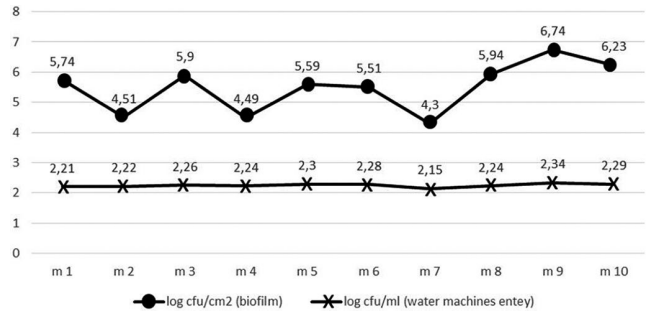

FIGURE 4 Bacterial count of dialysis water at the entry of the generators and the microbial load adhered in the pipes connecting the loop to the generators

3.2 | Bacterial identification results

The most frequent species of bacteria found in the water of the two sites studied (Table 1), were *Bacillus* and represent nearly the half of isolated strains (52%), followed by Enterobacteriaceae including *Enterobacter cloacae* 23, 52% in loop water and 17.39% in the water reaching the generators. Positive and negative Coagulase Staphylococci are also contaminants of dialysis water. Species often considered as markers are also present such as *Klebsiella pneumoniae*, *P aeruginosa*, *Acinitobacter baumanii*.

According to Table 1 and Figure 5, all these species were present in the two types of water analyzed except *A baumanii*, which was found only in the water taken at the entrance of the generators. The distribution of strains isolated in both types of water varied for each species. Some species of the genus *Bacillus* such as *Bacillus mycoides*, *Bacillus lentus*, *Bacillus lechiniformis*, *and Bacillus cereus* were found more at the entry of the generators. Also some species of Staphylococcus.

The species *Bacillus pumilis* was present in the loop water more than in the entry of the generators (nearly 9% higher).

The percentage of enterobacteria (*Enterobacter and Klebsiella*) decreased and was replaced by an increase of non-fermenting bacteria such as *P aeruginosa* and *A baumanii*.

Of these tubing segments (Table 1), 52 strains were isolated and purified. Among bacterial isolates, the genus *Bacillus* (21) was the most common with predominance of *B mycoides* and *B pumilis*. *The Staphylococci* spp were more regularly distributed. The enterobacteria (11) also found and mainly represented by *E cloacae* (07), 02 *Klebseilla pneumoniae*, 02 *Klebseilla oxytoca*. The other species present in this biofilm were *P aeruginosa* (07) and *A baumanii* (02).

3.3 | Microbial ecology results by dialysis machines

Each sectioned segment of the tubing had a different microbial ecology. The pipes of machines 8 and 9 were highly contaminated and their biofilm was composed of several species, followed by machines 10, 3, and 4 where the species of *B pumilis* was the most representative. *E cloacae*, *B mycoides*, *P aeruginosa*, *A baumanii*, and *S aureus* were also present (Figure 6).

Most species isolated from the biofilm were also present in the water taken at the entry of the dialysis machines. For some species, their presence was mainly in the biofilm namely *Klebseilla oxytoca*. The other species were found in both types of samples (water and biofilm).

Strains isolated from pipe segments showed a difference in biofilm formation capacity. Forty-two of fifty-two strains could form a biofilm, more than half (29) were highly formative, 13 strains with medium capacity and 10 were weakly biofilm-forming. Almost all strains isolated from the tubes of machines 9 and 8 were hyper-biofilm producers, including *P aeruginosa*, *A baumanii*, *E cloacae*, *and S aureus*.

4 | DISCUSSION

Many patients must be treated with renal replacement therapy for a long time because kidney transplantation is not common at Tlemcen University hospital (Algeria). Each week, patients are exposed to approximately 400 to 600 L of water during dialysis, with increased susceptibility to contamination.[15,16]

High concentrations of bacteria, including fragments of endotoxin, peptidoglycan, and bacterial deoxyribonucleic acid, can cross low and high flux membranes, stimulate cytokine production and set off the elevation of reaction proteins of acute phase such as C-reactive protein.[17]

Various microorganisms can multiply rapidly in various fluids that present in the water treatment system in a hemodialysis center, including water produced by distillation, deionization, reverse osmosis

TABLE 1 Isolated strains of loop water, at the entry of the generators and from tubing segments

	Loop water	% of genus	Water entry machines	% of genus	biofilm	% of genus
Bacillus pumilis	(15) 22,09%	51,49%	(6) 13,06%		(7) 13,46%	40,38%
Bacillus mycoides	(11) 16,17%		(8) 17,39%		(6) 11,54%	
Bacillus lentus	(5) 7,35%		(5) 10,86%	49,99%	(4) 7,69%	
Bacillus cereus	(3) 4,41%		(2) 4,34%		(3) 5,77%	
Bacillus lechiniformis	(1) 1,47%		(2) 4,34%		(1) 1,92%	
Staphylococcus epidermidis	(3) 4,41%	11,76%	(3) 6,55%	15,25%	(3) 5,77%	21,15%
Staphylococsus warneri	(3) 4,41%		(2) 4,36%		(4) 7,69%	
Staphylococcus aureus	(1) 1,47%		(1) 2,17%		(2) 3,85%	
Staphylococcus saprophyticus	(1) 1,47%		(1) 2,17%		(2) 3,85%	
Enterobacter cloacae	(16) 23,52%	23,52%	(8) 17,39%	17,39%	(7) 13,46%	13,23%
Klebseilla pneumoniae	(4) 5,88%	5,88%	(2) 4,34%	4,34%	(2) 3,85%	7,69%
Klebseilla oxytoca	(0) 0,00%		(0) 0,00%		(2) 3,85%	
Pseudomonas aeruginosa	(5) 7,35%	7,35%	(5) 10,86%	10,86%	(7) 13,46%	13,46%
Acinitobacter baumanii	(0) 0,00%	0%	(1) 2,17%	2,17%	(2) 3,85%	3,85%
Total	(68) 100%	100%	(46) 100%	100%	(52) 100%	100%

and softening. This kind of water is normally considered to be devoid of nutrients.

If the level of bacterial contamination exceeds the currently accepted limits, the result may be sepsis or endotoxemia by Gram-negative bacteria.

The dialysis water quality upstream of the dialysis machine is essential because it is used in dialysate preparation. This one will interact with the patient's blood through the dialysis membrane and for this reason many standards are proposed to establish the microbiological and chemical quality of the dialysis water; more widely known are the recommendations of the Association for the Advancement of Medical Instrumentation (AAMI 2004)[18] and those of the European Pharmacopoeia (2011).[19]

The microbiological recommendations of AAMI allow an upper limit of 200 CFU/mL for microbial contamination and 2 IU/mL for endotoxin contamination of water, the AAMI requires corrective actions of disinfection and cleaning beyond 50 CFU/mL for bacteria and 1 IU/mL for endotoxins. The recommendations of the European Pharmacopoeia set more restrictive limits: bacterial contamination <100 CFU/mL and endotoxins <0.25 IU/mL.[20]

Our counts (118 CFU/mL at the loop and 182 CFU/mL at the entry of the dialysis machines) show that the water used for dialysate preparation is contaminated, and exceeds the standards of the European Pharmacopoeia and that of the Ministry of Health in Algeria requiring a level of contamination below 100 CFU/mL. The microbiological quality of the water analyzed was well below the limits recommended by the IMAA.

Currently, concerning microbiological limits and cultivation techniques, in addition to the international standard AAMI[21] and the European Pharmacopoeia, an equivalent international standard[22] is currently under revision, proposes to reduce the two levels of bacteria and endotoxin levels in this new standard, published in 2009. These recommendations focus on culture techniques, particularly for bacteria: Tryptone Glucose Extract Agar (TGEA) and Reasoner Agar (R2A) at 17-23°C for 7 days. A recommended practice providing guidelines for the use, care, and/or treatment of a medical device or medical system is necessarily should not be a static document applied to a dynamic technology.

Counting bacteria on both R2A media and nutrient agar shows and proves that the enumeration of microorganisms that develop in

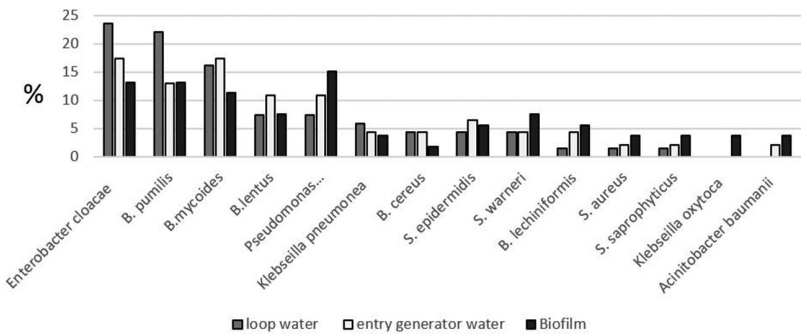

FIGURE 5 Percentages of strains isolated from loop water, biofilm and water entering the dialysis generators

	B. cereus	B. lechinformis	B. pumilis	B.lentus	B.mycoides	S. aureus	S. epidermidis	S. saprophyticus	S. warneri	E. cloacae	K. pneumoneo	K. oxytoca	P. aeruginosa	A. baumanii	Total
m 1				X O	X O					X			O		3 / 3
m 2	X	X O	O		X				O	O			X		4 / 4
m 3		O	X O		X		X	X O		X O			X O		6 / 5
m 4			X O		X O		X O			X			X O		5 / 4
m 5			X	X O						X O	X O		X O		5 / 4
m 6		X	O	X	X O			O		O		O			3 / 5
m 7			X O		X O		X O			X					4 / 3
m 8	X O	O	X O			O			X O	X O	X O	O	X O		6 / 9
m 9			O	X O	X O	X O			X O	X O			O	X O	6 / 8
m 10			X	X O	X O		O		O	X O			O	O	4 / 7
Total	1 / 2	2 / 3	6 / 7	5 / 4	8 / 6	1 / 2	3 / 3	1 / 2	2 / 4	8 / 7	2 / 2	0 / 2	5 / 7	1 / 2	46 / 52

FIGURE 6 Breakdown of identified strains in biofilm by machine. **X**: strains water entry machine; **O**: tubulure strains

extreme environments shows better laboratory culture results when they are incubated under conditions that simulate these environments. For this reason, the bacteria present in the dialysis water is better cultivated on nutrient-poor media, such as R2A and incubated for more than 96 hours and temperatures around 22°C.[8,20,23-26] In our case, the number of bacteria grown on R2A agar is 3 to 11 times higher than that of nutrient agar.

The comparison of our bacterial concentration results for the water samples, over a 5-month monitoring period with water arriving at the 10 dialysis machines included in this study, is shown in Figures 1 and 2. All water counts at the entry of the generators are more contaminated (182 CFU/mL on average) than that of the loop water (118 CFU/mL on average) with quantities greater than those allowed by the national standards of 100 CFU/mL. Twenty samples were analyzed and 17 exceeded 100 CFU/mL and all the samples taken at the entry of the machines exceeded the standards recommended by the European Pharmacopoeia.

The number of heterotrophic bacteria allowed by national standards in water is worrying because it is part of dialysate that promotes the growth of microorganisms which can release large amounts of endotoxin.

Similar studies give different results, and this is due to the microbial ecology of the centers studied and also due to differences in study methods.[17,27]

In this dialysis center, disinfection of the water treatment system is not done regularly, no procedure is documented.

According to service staff, the water purification systems of the service did not have a maintenance protocol for the water purification systems. These results show that water system managers are still unaware of the importance of regular maintenance and internal quality control of the water purification system.[27] Sometimes the supply of the disinfectant is not assured regularly, and every time there was a change of the disinfectant.

In most hemodialysis centers, staffs believe that the dialyzer filter provides total protection to patients, and does not allow any dangerous molecules to pass through patients' blood.

Water arriving at dialysis machines is significantly more contaminated than that of the loop, this can be explained by the growth of bacteria in biofilm, macroscopically visible in the form of a white coating on the inner walls of plastic pipes providing the connection between the loop and the dialysis machines. This state was confirmed by enumeration of adhered bacteria, where the level of contamination by machine could be 5.5 × 106 CFU/cm^2 or 25 000 times that of generator water 220 CFU/mL.

The pipes that connect the generators and the dialysis loop have the particular feature that was never being changed and disinfected, neither during the disinfection of the dialysis loop nor during the disinfection of the machine after each session.

Thenumberofadheredbacteriavariesfrom4.3to6.74logCFU/cm^2; these tubules are nearly 2 m long and the number of bacteria adhered is at the order of billions. This high level of contamination is a health hazard for dialysis patients.

Thus, the water in these pipes is stagnated when the machines are stopped posing a real problem in the system of dialysis water treatment. The introduction of a new process for the regular and efficient disinfection of this area is therefore essential for the hygienic maintenance of the water treatment units to maintain the bacterial count values below the action level and therefore a better hemodialysis quality.[28]

All surfaces in the hemodialysis fluid pathways are susceptible to biofilm invasion.[14,29-31] The importance and speed of biofilm growth will depend on three main factors: types of materials; the design of the distribution system, including flow rates and dead zones; and the frequency and effectiveness of disinfection treatments.[29]

Very few studies are aimed at biofilms in hemodialysis systems. However, studies have been conducted and agree that biofilms are a major problem for which routine disinfection treatments are ineffective.[32-34]

This suggests that despite the routine disinfection procedure, biofilms develop in the hydraulic circuit of the water treatment system and/or hemodialysis monitors. Nystrand was reported that a microbial count result in a system, that is regularly disinfected because of somewhere in the system, microbial growth is current, has been shown that the presence of a biofilm on the pipes led to rapid regrowth of the bacteria after a few hours of disinfection of the water supply system.[17,35]

The arrival water to dialysis machines is very contaminated by bacteria where they will find a place with adequate pH, a temperature of 37°C and glucose. With all these factors and an average period of 3 hours for each dialysis session, the bacteria multiply rapidly and considerably.

Each dialysis system of water production has a particular microbial ecology, considering the difference in the source of water, the general state of hygiene of the reservoirs,the treatments undergone, the degree and frequency of disinfection.[36]

The identification ofbacteria present in the dialysis water (loop and generators) shows the major presence of bacteria commonly found in water such as *Bacillus* and coliforms including *E cloacae*.[8] The bacterial species found in our study are also identified in other similar studies. What seems worrying is not only the presence of marker and pathogenic bacteria such as *P aeruginosa*, *A baumanii*,

and S aureus in the water of the generators and consequently the dialysate, which can cause serious infections at dialyzed patients by releasing of toxins, but also their ability to live in biofilm because almost all are biofilm-forming and resistant to all disinfectants,[8] and are source of endotoxins which present a real threat in the dialysis process.[8,37,38]

Several Gram-negative bacilli have been isolated from the water distribution system and dialysate. We isolated Pseudomonas genus at 52.8% in agreement with other authors.[17]

5 | CONCLUSION

The water quality in dialysis units is influenced by the colonization rate and the presence of biofilm in the distribution system. Therefore, the use of disinfection methods, recommended by the standards, prevents the occurrence of infections and endotoxin shock of hemodialysis patients.

Differences in standards in terms of microbiological quality of dialysis water remain a dubious point that favors a lot of deviation. Microbiological standards must be strict and uniform.

Also a germ-free and endotoxin-free dialysate does not exclude the risks. The risks of bacterial and endotoxin discharge from the biofilm developed on the circuit tubing, acting as a reservoir for continuous contamination.

We believe that cleaning, disinfection optimization efforts, and the procedures used for hemodialysis systems should aim to detach and neutralize the biofilm when necessary.

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