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Original article

Microbiological monitoring of ultrapure dialysis fluid in a hemodialysis center in Alexandria, Egypt

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1. Introduction

Microbiological contamination of dialysate is an issue that attracted attention since the 1990s. This is partly due to the extensive use of sodium bicarbonate as a dialysate buffer, which helps microbial proliferation, and also the use of high flux dialysers that allow back filtration of microbial contaminants.¹ Microbial contaminants most frequently found in dialysis water are bacteria and their degradation products, such as endotoxins, peptidoglycans and bacterial DNA (bDNA). Fungi, viruses and protozoa can also be encountered occasionally.² Endotoxins are heat-stable lipopolysaccharides (LPS) and the major cell wall components of Gram-negative bacteria.³ The molecular mass of LPS ranges between 2000 and 20,000 Da. LPS can be transferred through membranes with large pore size by back filtration/diffusion from the dialysis fluid to the blood compartment.⁴ If the dialyser membrane is tight, bacteria are not able to pass from the dialysate compartment into the blood compartment. However, endotoxins, can permeate and cause an inflammatory state.⁵

Exposure to microbial contaminants is clearly associated with short-term complications, ranging from pyrogenic reactions to septicaemia.^{6,7} Furthermore, microbial contamination of dialysis fluid is an important cause of chronic inflammation and MIA syndrome (malnutrition, inflammation, and atherosclerosis) among end-stage renal disease patients. Long-term HD patients suffer from arteriosclerosis, and β_2 -microglobulin-associated amyloidosis with complicating spinal stenosis, carpal tunnel syndrome, bone cysts and arthropathy. To overcome the above-mentioned short and long-term complications of microbiologically

contaminated dialysis fluid, the use of ultrapure dialysis fluid (UPDF) which has a lower microbial load was recommended. This has been accompanied by an improvement in the inflammatory state of HD patient.⁸

The Association for the Advancement of Medical Instrumentation (AAMI) and the European Pharmacopoeia (EP) established upper limits for microbial contamination of UPDF as containing <0.1 colony forming unit (CFU)/ml and <0.03 endotoxin unit (EU)/ml using LAL assay.⁹ To produce highly purified (ultrapure) water, a system is required based on having a second reverse osmosis (RO) module and/or an electrochemical deionizer (DI) placed in series.¹⁰ There are no international standards for fungi in dialysis fluids, however Fresenius Medical Care (FMC) recommended the level of fungi to be <10 CFU/ml in dialysis water and dialysis fluid, and <1 CFU/ml in UPDF.¹¹

2. Aim of the work

This study aimed at evaluating microbial contamination of dialysate and UPDF through estimation of heterotrophic plate count, total coliforms and fungi as well as endotoxin detection.

3. Material and methods

This cross-sectional study was carried out during a 6-months period, on a total of 100 dialysis fluid samples randomly collected from a private HD center in Alexandria, Egypt. The HD unit consists of one water treatment unit and two rooms for HD. In this unit, water passes through a RO filter and a mixed bed DI, and then it is stored in a tank of 1000 Liter capacity. The finally treated water is pumped into two rooms of HD through a system of polyvinyl chloride (PVC) pipes to reach every HD machine. Chlorination is done every month for maintenance of water treatment unit and the bacterial filter (0.2 μ m) is substituted every month. The total

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number of HD machines in both HD rooms is eight machines. The first room is back to back to the water treatment unit and contains three machines. On the other hand, there is a long distance (about 20 m) between the water treatment unit and the second room which contains five machines. Most of the machines were commercial type A, the remaining were type B which is relatively newer version with some technological advances. The dialysate concentrate used in this unit is a sealed sterile capsulated bicarbonate powder along with the complementary solution A for bicarbonate based HD (referred to as A-component). Chemical disinfection of machines is done after every session using 0.2% peracetic acid solution 30 min at room temperature.

3.1. Sampling

A total of eighty UPDF (infusate) samples were collected from all online hemodiafiltration (HDF) sessions. In addition, twenty pure dialysis fluid samples (distributed as 10 dialysis water and 10 final dialysate samples) were collected on weekly basis. Samples were aseptically collected in sterile wide-mouthed glass containers (1000 ml containers for infusate samples, 900 ml each, and 500 ml containers for dialysis fluid samples, 300 ml each).¹²

Thirty UPDF samples were collected from room (1) and 50 samples from room (2), out of which, 10 samples were from Type B machine.

Before collecting the final treated water samples (dialysis water), the faucets were wiped with alcohol, left to dry and then water was left to flow for one minute to flush any residuals in the connections and pipes. The final dialysate and the infusate fluid samples were collected from sampling ports by allowing the fluid to flow for half a minute then aseptically collected in glass containers. The samples for endotoxin analysis were collected aseptically by one ml depyrogenated sterile disposable syringes.

All the collected samples were labeled, transported and delivered to the laboratory as soon as possible to be analyzed within 1–2 h of collection. In case of expected delay, samples were stored at 4 °C and analyzed within 24 h maximum. Each sample was accompanied by a sheet including: sampling date, time of collection, sampling site and type of machine. All samples (dialysis water, final dialysate and infusate) were subjected to membrane filtration method, the membranes were plated onto the following media: Reasoner's 2A (R2A) agar (Oxoid CM0906), Eosine Methylene Blue agar (EMB) (Levine CM0069) and Sabouraud dextrose agar (SDA) (Oxoid CM0041) supplemented with 50 mg chloramphenicol/liter. R2A, a low nutrient medium used for the recovery of stressed heterotrophic bacteria found in high purity water, was used as recommended for HPC, and EMB was used for detection of total coliforms while SDA was used for fungal cultivation. R2A and SDA plates were incubated at room temperature (25 °C) for at least 5 days and up to 7 days, while EMB plates were incubated at 37 °C for 24 h. After the incubation period, colonies were counted and reported as CFU per ml of sample. LAL assay for endotoxin measurement was done by gel clot method, with Pyrotell

(Associates of Cape Cod, Inc.) of sensitivities 0.25 EU/ml and 0.03 EU/ml for pure and ultrapure water samples respectively.

During the sessions in which samples were collected observation of any febrile/ pyrogenic reactions in patients were recorded.

3.2. Statistical analysis

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. Qualitative data were described using number and percent. Quantitative data were described using range (minimum and maximum), mean, standard deviation and median. Significance of the obtained results was judged at the 5% level.

4. Results

During the time of sample collection, none of the HD patients encountered any febrile/pyrogenic reaction.

According to the European standards for HD fluids and Fresenius Medical Care (FMC) guidelines^{11,13} the final treated water and the final dialysate were considered unacceptable if one or more of the following criteria were found: HPC > 100 CFU/ml, presence of coliforms, endotoxin level > 0.25 EU/ml and fungi > 10 CFU/ml. On the other hand, the infusate was considered unacceptable if one or more of the following criteria were found: HPC > 0.1 CFU/ml, presence of coliforms, endotoxin level > 0.03 EU/ml or fungi > 1 CFU/ml.

According to the above standards, TC and fungi parameters were found acceptable in all samples (100.0% each). Out of 80 infusate samples, 63 samples (78.8%) were acceptable when examined for HPC alone. The percentage of acceptability decreased to 35.0% when examined by LAL alone and further decreased to 28.8% when collectively evaluated by all parameters (Table 1).

The mean count of HPC was recorded for the infusate samples as 0.09 ± 0.11 CFU/ml, and for dialysate and dialysis water samples as $(16.8 \pm 2.39, \text{ and } 23.0 \pm 2.54)$ CFU/ml, respectively. Nearly equal mean counts of fungi were present in infusate, dialysate and dialysis water samples ($0.05 \pm 0.09, 0.04 \pm 0.02$ and 0.05 ± 0.04 CFU/ml respectively), and all were within acceptable levels (Table 1).

Concerning the two types of HD machines included in the study, the mean count of fungi of infusate samples from Type A machines was higher than that from Type B ($0.05 \pm 0.10, 0.02 \pm 0.01$ CFU/ml) respectively. However, the difference was statistically insignificant ($p = 0.329$). On the other hand, infusate samples from Type A machines had significantly higher mean HPC counts than those from Type B ($0.09 \pm 0.12, 0.02 \pm 0.02$ CFU/ml) respectively ($z = -3.60, p = 0.000^*$).

Out of 70 samples from Type A machines, 18 (25.7%) were acceptable and 52 (74.3%) were unacceptable for endotoxin by LAL testing. On the other hand, all the 10 samples from Type B machine were acceptable by LAL, and this difference between both machines as regards LAL testing was found to be statistically significant ($p = 0.000$) Table 2.

Table 1
Distribution of the acceptability of the 100 HD fluids samples according to the examined microbiological parameters.

Parameter examined	Infusate samples (80)			Dialysate samples (10)			Dialysis water samples (10)		
	Standard	Acceptable		Standard	Acceptable		Standard	Acceptable	
		No.	%		No.	%		No.	%
HPC	<0.1 CFU/ml	63	78.8	<100 CFU/ml	10	100.0	<100 CFU/ml	10	100.0
LAL	<0.03 EU/ml	28	35.0	<0.25 EU/ml	9	90.0	<0.25 EU/ml	10	100.0
TC	Zero	80	100.0	Zero	10	100.0	Zero	10	100.0
Fungi	<1 CFU/ml	80	100.0	<10 CFU/ml	10	100.0	<10 CFU/ml	10	100.0
Total acceptability		23	28.8		9	90.0		10	100.0

Table 2
Distribution of infusate samples according to HD machine type and LAL acceptability.

Machine type	Examined samples (80)	LAL				P
		Acceptable ≤0.03 EU/ml		Unacceptable >0.03 EU/ml		
		No.	%	No.	%	
Type A	70	18	25.7	52	74.3	0.000 [*]
Type B	10	10	100.0	0	0.0	

p: p value of Fisher's exact test.

^{*} Statistically significant at $p \leq 0.05$.

Table 3
Acceptability of samples from Type A machines according to HPC and LAL parameters and its relation to the total working hours of dialysis machines.

Total working hours	Examined samples (78) ^{**}	Acceptable samples for HPC		Acceptable samples for LAL	
		No.	%	No.	%
<5000	29	23	79.3	15	51.7
5000–10,000	40	32	80.0	9	22.5
>10,000	9	6	66.7	1	11.1
Test of significance (χ^2)		0.799		8.641	
(P- value)		0.671		0.013 [*]	

^{*} Statistically significant at $p \leq 0.05$.

^{**} 78 = 70 infusate and 8 dialysate samples.

Table 4
The relationship between LAL and HPC parameters among the 80 examined infusate samples.

HPC	LAL		Total
	Acceptable <0.03 EU/ml	Unacceptable >0.03 EU/ml	
Acceptable	23	40	63
Unacceptable	5	12	17
Total	28	52	80

Observed agreement: 0.44, Z = 0.96.

Concerning the 2 rooms of HD, infusate samples from Type A machines were compared in both rooms. There was an insignificant difference between Room 1 and Room 2 as regards HPC and fungal counts (HPC: 0.08 ± 0.06 CFU/ml and 0.11 ± 0.14 CFU/ml respectively, fungal count: 0.07 ± 0.13 CFU/ml and 0.04 ± 0.08 CFU/ml respectively). However, Room 1 showed higher percentage of acceptable infusate samples from Type A machines by LAL test (53.3%) than Room 2 (5%) with statistical significance ($\chi^2 = 20.96$, $p = 0.000$).

About 80% of samples from Type A machines with a total working hours <5000 or 5000–10,000 were acceptable for HPC compared to 66.7% of samples from machines with >10,000 h. However, this difference was statistically insignificant. On the other hand, when examined by LAL, the percentages of acceptability decreased to 51.7% and 22.5% for the machines with total working hours <5000 and 5000–10,000 respectively, compared to 11.1% for those with >10,000 h. This difference was statistically significant ($\chi^2 = 8.6$, $p = 0.013$) (Table 3).

Out of the 80 infusate samples, 63 samples were acceptable by HPC, and only 23 of these were also acceptable by LAL (Table 4). The observed agreement between both parameters (HPC and LAL) was 44%.

5. Discussion

The quality of the dialysate is of paramount importance in assuring patient safety. To help prevent injury to HD patients from contaminants in the dialysate, standards have been developed for the quality of the dialysate as well as the water concentrates and devices used in its preparation¹³.

Several studies reported the presence of microbiological contaminants in dialysate^{14–18}; however, data on ultrapure contamination are infrequently available. In the present study, although the acceptability for infusate was poor regarding both HPC and LAL assay parameters, the respective acceptability for treated water as well as dialysate was almost 100.0%. This can be explained by the more stringent standard for the infusate. This high standard was not feasible to be accomplished by the older machine technology (type A) which was of less technological advances. Whereas recent advances in machine technology (type B) could compensate for any potential defect in water treatment unit raising the acceptability of the infusate again to 100.0%.

In this study, the observed discrepancy between LAL assay and HPC parameter agrees with reports of Klein et al.¹⁴ Kulander et al.¹⁵ and Bland et al.¹⁶ who found no correlation between bacterial growth and endotoxin concentrations in water or dialysate samples. They proposed that low levels of bacterial growth in the dialysate might be associated with high endotoxin concentration, since endotoxin is released mainly after death of microorganisms. Ledebø and Nystard recommended that endotoxin levels should always be measured in addition to bacterial counts because they give a different yet complementary picture of the microbiological quality of the fluid.¹⁷

Regarding final dialysate, the present study revealed 100.0% acceptability for HPC, while El-Koraie et al. reported lower compliance results (51.7% and 86.1% acceptability at two HD units in Alexandria).¹⁸ The AAMI/ISO standards recommend culturing conditions that more closely resemble the conditions under which contaminating bacteria exist in the systems used to prepare dialysate. These new culturing conditions have been shown to increase the yield of bacteria obtained for a given water or dialysate sample, thus providing better information on the level of contamination in the system.^{9,19} The above mentioned culturing conditions have been implemented in the current study. In addition, the MF method is also recommended by the AAMI/ISO standards and has been implemented in this study for detecting low levels of contamination.

There are few reports concerning the extent of fungal contamination of dialysis fluid. Figel et al.²⁰ isolated black fungi from the municipal supply and in treated water for dialysis and dialysate. Most of the international standards do not include fungi as

indicators of quality for dialysis, except in Sweden, where the current standards for fungi are 10 CFU/ml.²⁰ In this piece of work, no fungal elements above the standard level were recorded.

In this study, TC were not isolated in any of the samples, where dialysate concentrate used in this unit is a sealed sterile capsulated bicarbonate powder (ready-made Bicart capsules). However, in a previous study conducted in Alexandria, TC were isolated from 4% of examined samples, where reconstituted bicarbonate powder was used, which is more liable for contamination. In that same study, one sample was contaminated with *E. coli*, but neither TC nor *E. coli* were revealed from the unit that was using ready-made Bicart capsules.¹⁸ Again, Zunino et al.²¹ encountered TC in 0.5% of samples whereas Arvanitidou et al.²² reported much higher results: 12.3% for TC and 8.6% for *E. coli*.

Water purification by a double RO module is recommended, because microbiological water quality after purification with a single RO does not often comply with the reference quality level.²⁴ The presence of a treated water-storage tank, as in the current study, which may not be frequently drained and disinfected, is a recognized factor contributing to persistent bacterial contamination and to biofilm formation.^{6,9} Favero et al. stated that storage tanks should not be used unless provision were made for frequent draining and adequate disinfection.²³ Concerning the distribution system, high grade stainless steel is the only material resistant to biological corrosion.¹⁰ However, PVC is usually used because of its low cost, as in this studied dialysis unit.

In the present study, infusate samples were collected from two dialysis rooms, the first (room 1) is back to back to the water treatment unit, while for the second room, long distribution tubes were encountered (about 20 m long). This could explain the reason for the much higher percentage of acceptable LAL infusate samples at room 1 than room 2 (53.3% and 5.0% respectively). In addition, HPC mean counts of infusate samples collected from dialysis machines at room 2 were higher than those at room 1 (0.11 and 0.08 CFU/ml respectively), although did not reach a significant level. These data indicate that dialysis fluid pathways should be configured in a loop, be as short as possible, have no dead-ends, and contain the minimum number of pipe fittings and joints.^{9,19} Otherwise, biofilm formation is frequently encountered in such points, allowing the shedding of bacteria in low counts and the continuous release of their degradation products, as the endotoxins.

Regarding the finally treated water, the present study revealed a 100.0% HPC acceptability. There was a significant association between the quality of final dialysate and that of treated water. Similarly, a study conducted by El-Koraie et al.¹⁸ showed an association between the final dialysate and each treated water and concentrates in the two examined HD units. In contrast, Baumbauer et al.²⁴ found no correlation between the level of contamination of dialysate and the water processing method or type of concentrate.

Arvanitidou et al.²² suggested that the increasing working hours of dialysis machines correlated more with the quality of dialysate, as it becomes more difficult to clean or disinfect. In accordance with those workers, the present study has shown significant decrease of acceptability for LAL parameter with the increase of the total working hours of the Type A machines.

Pertaining to HD machines, this study demonstrated significant differences between Type A and Type B machines in the mean counts of HPC and in LAL test results of infusate samples. This might be related to the fact that Type B machine is of newer generation which incorporates recent technology regarding continuous flow of dialysis fluid, the heating system and avoidance of closed circuits and loops. Of note, that the back filter in the Type B machine is a double filter bath (diasafe), whereas in the Type A machine it is only single (foreclean filter). New machines have

incorporated several recommended features including low volumes of hydraulic circuit, high dialysate rates, global disinfection and non stagnant zones.¹⁰

Fortunately, despite the detection of endotoxin levels that were more than the permissible one (0.03 EU/ml), there were no encountering of any pyrogenic reactions among patients on HDF sessions. This could be considered as potential limitation, as the main aim of this study did not address clinical signs for endotoxemia and inflammatory reactions, which can be further investigated in future research. However, this issue was previously tackled in assessing endotoxin level in HD patients and its role in inducing inflammation.²⁵ Di Iorio et al. reported after a one year study of using ultrafilter, C-reactive protein and pro-inflammatory cytokines decreased while anti-inflammatory ones increased, and hemoglobin levels were improved. They speculated that ultrapure water can ameliorate the inflammatory status and improve survival of dialysis patients.²⁶

The present study has shown 44% agreement between HPC and LAL parameters performed to assess the quality of the infusate. The same percentage of agreement was reported by El-Koraie et al. in Alexandria.¹⁸ On further analysis of our findings, HPC showed 21.2% unacceptability, which increased to 65.0% and 71% by LAL and by both HPC and LAL respectively. In accordance with these findings, El-Koraie et al. revealed that HPC showed 40.0% unacceptability, which increased to 88.0% and 92.0% by LAL and by both HPC and LAL respectively.¹⁸

6. Conclusion

This study highlighted the potential risk and safety concern that might be associated with the use of online HDF if to be generalized without implementing the international microbiological standards for ultrapure dialysate quality. Again, the ability to reach a 100.0% acceptability of the infusate using the more advanced newer HDF machines can raise the hope of implementing such technique with a high safety margin. It is also evident that long distribution tubes in the HD unit, as well as long working hours of HD machines, are of paramount importance in determining the quality of infusate used in online HDF.

Conflicts of interest

All authors declare that they have no conflicts of interest in relation to the present manuscript.

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Declaration of interest

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