Dialysis water and fluid purity: more than endotoxin

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Abstract

The evolution of extracorporeal treatment of end-stage renal failure has enforced focus on the purity of dialysis fluid. A major challenge of high-flux haemodialysis (HD) and haemodiafiltration relates to the necessity for ultrapure dialysis fluid and for sterile non-pyrogenic substitution fluid. The present review focuses especially on the possible microbial contamination including, next to intact micro-organisms, a variety of microbial derivatives. It is pointed out that there are conditions (e.g. contamination by non-culturale micro-organisms or bacterial derivatives other than lipopolysaccharides) where the detection of biologically relevant contaminants can be missed when applying the recommended standard detection methods such as bacterial culture and limulus amoebocyte lysate test. Possible approaches for action upon positive sampling results, exceeding the limits recommended in the latest ISO 11663:2009, are described in detail and illustrated with flow charts. The issue of purity of dialysis fluids is highly relevant, since the chronic exposure of HD patients to low levels of cytokine-inducing microbial components can significantly contribute to the micro-inflammatory status of these patients.

Keywords: approaches for action; detection methods; dialysis fluid purity; microbial contamination; purity standards

Introduction

The evolution of extracorporeal treatment of end-stage renal failure over the past decades, from haemodialysis (HD) with low-flux membranes to high-flux membranes followed by the introduction of on-line haemodiafiltration (HDF), has enforced focus on the purity of dialysis fluid. High-flux HD is currently applied in almost 50% of the patients on HD worldwide [1]. High-flux membranes were introduced in an attempt to reduce the morbidity and mortality of HD patients offering better biocompatibility and removal of larger uremic retention solutes. It soon became clear that even if limited to the HD mode, these membranes allowed convection as well [2]. Nevertheless, outcome benefits could only be found in secondary or subgroup analyses of the two available randomized trials comparing high-flux versus low-flux membranes, the haemodialysis (HEMO) study [3, 4] and the membrane permeability outcome (MPO) study [5]. In the HEMO study, a survival benefit of high-flux membranes was found for patients on HD for more than 3.7 years [3]; in addition, a decreased risk of cardiac deaths and the composite of cardiac deaths and the first cardiac hospitalization [3] and death from previously unknown cerebrovascular disease were suggested [4]. In the MPO study, a significantly higher survival rate for patient subgroups with a serum albumin below 4 g/dL and for diabetic patients was

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observed when they were treated with high- compared with low-flux membranes [5].

High-flux membranes, however, are only used at their optimal potential in a HDF setting allowing high fluid flows and convective transport, providing the largest removal of the widest range of uraemic retention solutes [2]. Two very recent controlled trials compared on-line HDF with either low-flux (contrast) [6] or high-flux HD (Turkish) [7]. In both studies, no differences could be observed at primary analysis. Only at secondary analysis, a lower mortality risk was shown in one-third of the HDF-treated patients who reached the highest convection volume of ≥20 L. These findings will need confirmation since data obtained by post hoc analysis are subject to inherent limitations and vulnerable to bias.

A major challenge of high-flux HD and HDF relates to the necessity for (i) ultrapure dialysis fluid, since contaminants can enter from the dialysis fluid into the blood by either convective transfer (backfiltration) or movements down the concentration gradient (backdiffusion), and (ii) sterile non-pyrogenic substitution fluid because otherwise contaminants may be infused directly into the circulation.

The present review will focus on general and specific aspects related to dialysis water and fluid purity, describing biological aspects of the possible contaminants and their relevance. In addition, the national and international recommended standards together with recommendations concerning sampling, and detection methods and strategies, will be discussed.

**Biological characteristics of contaminants**

When considering the possible contaminants which are present in water used to prepare dialysis fluid, we can distinguish three types: (i) particles, (ii) chemicals and (iii) microbial contamination. The present review will especially focus on the latter. Next to intact microorganisms such as bacteria, fungi and yeasts, a large variety of microbial derivatives or fragments can be released into any fluid during active growth and lysis of the microorganisms. The issue of purity of dialysis fluid is highly relevant, since the chronic exposure of HD patients to low levels of cytokine-inducing microbial components can significantly contribute to the micro-inflammatory status of these patients.

**Intact micro-organisms**

The water used for the preparation of dialysis fluid is not sterile at the origin: therefore, the micro-organisms present must be kept well under control, preventing bacterial proliferation in order to limit biofouling. In a study by Bambauer et al., evaluating, in 1994, the microbiological quality of water and dialysis fluid in 30 German HD centres, bacteria of the genus *Pseudomonas* [Gram negative and source of endotoxin (see below)] were found to be most prevalent in both water and dialysis fluid; however, Gram-positive micro-organisms [source of peptidoglycan (see below)] from the genera *Micrococcus* and *Staphylococcus* were also identified [8]. More recently, with more sophisticated bacterial identification techniques (also see below), HD water was shown to predominantly contain Gram-negative bacteria of the classes Alphaproteobacteria and Betaproteobacteria, as well as Mycobacteria [9].

When the number of bacteria becomes too high, viable microorganisms can switch from a planktonic (i.e. floating freely in the water environment) to a sessile (i.e. permanently attached) lifestyle, this process can be quantified by the determination of their cell density triggered by small water-soluble molecules (autoinducers such as thiolactones), a process called ‘quorum sensing’, which leads to biofilm formation [10]. During this process, bacteria attach to surfaces, which in the case of the dialysis fluid preparation circuit, are usually the reverse osmosis (RO) membranes, the distribution pipelines or dialysis monitor tubings and filters. Once attached, they encase themselves in a hydrated matrix of polysaccharides and proteins and form a slimy biofilm layer. An established biofilm causes recurrent contamination and is very difficult to remove with the current disinfection procedures [11]. An adequate design of a water treatment and distribution system is, for example, water pre-treatment followed by an RO module combined with a second RO and/or electrodeionizer, avoiding branches, dead ends and storage tanks, if possible. Such a set-up then still needs regular disinfection (heat or ozone) including the distribution loop and storage tanks, if applicable, and the microbial monitoring of the dialysis water treatment and distribution system to prevent biofilm formation [12, 13].

**Bacterial derivatives**

**Lipopolysaccharides and fragments of lipopolysaccharides.** Endotoxins (ETs) are heat-stable lipopolysaccharides (LPSs) and the major cell wall components of Gram-negative bacteria. The LPS molecules (Figure 1A) consist of a hydrophobic moiety, lipid A and an O-specific polysaccharide side chain covalently linked to lipid A via an inner core oligosaccharide of limited structural variability [14]. The molecular mass of LPS ranges between 2000 and 20 000 Da, although larger aggregates, called micelles, can easily be formed. In addition, the structural heterogeneity of LPS is large, which is reflected by differences in biological activity [15]. LPS has been shown to contaminate dialysis fluid at levels up to 2 endotoxin units (EU)/mL [16] or even higher up to 7.68 EU/mL (see ‘National and international standards for dialysis water and fluid purity’ and Table 1 below) [17]. This can cause inflammatory problems, since LPS can be transferred through membranes with large pore size by backfiltration/backdiffusion from the dialysis fluid to the blood compartment [18].

**Peptidoglycan.** The peptidoglycan (PG) or the murein layer is one of the main components of the bacterial cell wall. Chemically, PGs are complex heteropolymers composed of long glycan chains that are cross-linked by short peptides. The glycan chains consist of alternating N-acetylglucosamine and N-acetylmuramic acid residues
which are linked via β1-4-glycosidic bonds (Figure 1B) [19]. The chemical composition of the PG layer is similar in both Gram-positive and Gram-negative bacteria, but in Gram-positives, the PG layer is thicker and more cross-linked than in Gram-negatives. Contamination of dialysis fluids by PGs has mainly been described a few years ago.

Fig. 1. (A) The origin of different bacterial derivatives: LPS and bDNA fragments. (B) The origin of different bacterial derivatives: PG.
in the context of peritoneal dialysis when they were the cause of an epidemic of aseptic peritonitis in patients treated with icodextrin dialysis fluid [20]. Although these fluids met the purity levels prescribed by the pharmacopoeia with regard to the bacterial count and the ET level, they provoked an inflammatory response, pointing out that the classically used methods for the detection of contamination are insufficient to trace several types of bacterial derivatives [17]. In HD, PG levels of 4.1 ± 6.1 ng/mL in dialysis fluid from a central supply system were described [21], while in our own study evaluating PG levels in dialysis fluid collected from different units, PG levels up to 20 ng/mL were detected [17]. To the best of our knowledge, no membrane transfer data are available for PG, probably because the detection of these contaminants, even while they are biologically active, is missed with the classically recommended methods (see ‘Methods for the detection of microbiologic contaminants’ below).

**Short bacterial DNA fragments.** Compared with vertebrate genomic DNA, bacterial DNA (bDNA) contains more unmethylated deoxyctosine–deoxyguanosine phosphate (CpG) motifs, which activate the host defence mechanisms and lead to the innate and acquired immune responses. bDNA has been detected in RO water and HD fluid (0.28 ± 0.02 µg/mL), essentially because it is only partially removed by ultrafiltration [22]. It can diffuse through high-flux membranes and might be an overlooked factor contributing to inflammation in HD patients [22]. bDNA enhances the production of cytokines by mononuclear cells of chronic kidney disease patients and promotes the cellular survival of these pro-inflammatory cells by reducing apoptosis [23]. The same group demonstrated that bDNA stimulates the inflammatory response of CD14+CD16+ monocytes, which in addition are characterized by an increased number in HD patients; and the released inflammatory factors cause endothelial cell apoptosis [24]. Circulating levels of bDNA are associated with higher levels of C-reactive protein and interleukin (IL)-6 in HD patients [25].

**Toll-like receptor response**

The derivatives described above are pathogen-associated molecular patterns (PAMPs), which are recognized by pattern-recognition molecules (PRMs). Toll-like receptors (TLRs) function as PRMs and signal transducers in mammals and are expressed on monocytes, dendritic cells, vascular endothelial cells, adipocytes and intestinal epithelial cells [26]. TLR2 recognizes a variety of microbial derivatives such as LPS, PG and zymosan, whereas TLR4 is essential for signalling in response to LPS from Gram-negative bacteria, and TLR9 signalling is mediated by CpG DNA [26]. All these recognition systems can induce cell signalling pathways including the mitogen-activated protein kinases and nuclear factor κ-B resulting in cytokine production. All the above-described PAMPs, which can occur simultaneously in dialysis fluid, can exert synergistic effects, further increasing the inflammatory response to levels which are much higher than the expected sum of effects for the specific concentration of each single contaminant [27].

**National and international standards for dialysis water and fluid purity**

Over the years, many official recommendations for microbiological water quality have been published by both national authorities as well as international organizations, creating a confusing situation [28]. The most widely spread recommendation has been the ANSI/AAMI (American National Standards Institute/Association for Advancement of Medical Instrumentation) RD 52 (2004) according to which bacterial growth should not exceed 200 colony forming unit (CFU)/mL and 2 EU/mL for ET in water used to prepare dialysis fluids and in the standard

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EBPG, European Best Practice Guidelines; ANSI/AAMI, American National Standards Institute/Association for Advancement of Medical Instrumentation; ISO, International Organization for Standardization; CFU, colony forming unit; EU, endotoxin units; AL, Alert (AAMI) or Action (ISO) level; Sterility Assurance Level (SAL) 10⁻⁶, less than one chance in a million that viable micro-organisms will be present.
dialysis fluid per se. Standards for ultrapure dialysis fluid (0.1 CFU/mL and 0.03 EU/mL) and on-line substitution fluid (<10⁻⁶ CFU/mL and <0.03 EU/mL), however, are more strict. In addition, alert or action levels for pure water and standard dialysis fluid (50 CFU/mL and 1 EU/mL) have been proposed [29]. These are levels at which action (e.g. extra disinfection) should be undertaken to interrupt a trend towards higher unacceptable levels. It is of note that the standards for dialysis water (<100 CFU/mL and <0.250 EU/mL) recommended by the European Pharmacopoeia, adopted by the European Best Practice Guidelines (EBPG; currently ERBP—European Renal Best Practice) published in 2002 [30], were already far below the ANSI/AAMI standards published in the same year and even the revised ones from 2004 [29]. A need for global standards is desirable and this gap could now be filled by the International Organization for Standardization with ISO 11663:2009 and ISO 23500:2011 [31, 32].

Methods for the detection of microbiological contaminants

The reliability of any detection method starts with a correct sample collection at the outlet of the water distribution system (e.g. downstream the RO and, if possible, at the end of the loop) or prior to the dialyser for dialysis fluid. Prior to sampling and as summarized in Figure 2, the outlet system (sample port or connector) should be disinfected using 70% ethanol or isopropanol, with an exposure time of ∼30 s. From our own experience, installation of fixed sample ports, if they are subjected to continuous water flow (no stagnation), are the best option, since they avoid repeated opening of the circuit. Sample recipients should be sterile and ET-free. The first sample of ∼20 mL should be discarded and a fresh sample should be taken using a new syringe. For bacterial culture (BC), sample volume depends on the sensitivity needed and can vary between 10 and 1000 mL, whereby membrane filtration can help to increase sensitivity (see below). For ET quantification and bioassay, a sample volume of 5 mL each will suffice. Samples should be stored at 4°C for a maximum of 24 h and preferably be

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**Fig. 2.** Sampling recommendations and microbial detection tools.
processed as soon as possible. Under exceptional circumstances (longer expected transportation times, unforeseen evaluation delays), samples can be frozen although not for BC purposes.

**Intact micro-organisms**

In order to form visible colonies for the determination of the number of CFUs, micro-organisms must have the possibility to grow under the applied culture conditions (medium, temperature and incubation time). Preferable culture conditions for water-borne micro-organisms have been described in several national and international recommendations (AAMI; ERBP, ISO). Culture media should be nutrient poor, e.g. Reasoner’s Agar n°2 (R2A) or tryptone glucose yeast extract agar (TGEA). A prolonged culture time of 7 days is required, as is incubation at room temperature (17°C–23°C). Although this remains the recommended and most practical approach, one should be aware that these conditions are not always favourable for growth of each micro-organism and as estimated, only 30% of the micro-organisms present will grow [33]. So, there is a need for more sensitive methods than the ones currently available [28]. More advanced techniques like a molecular culture-independent approach based on the analysis of the 16S rDNA isolated from total bDNA from lysed bacteria after dialysis water or fluid filtration are very sensitive and could offer an indication of the type of micro-organisms present [9], but not of their number. It is of note that the availability of the technique might be restricted, while the reference libraries are too limited. Recently, an electrical detection method using antimicrobial peptides immobilized on a micro-electrode array has been described. This device could be used in an on-line setting if its sensitivity could be improved so as to meet the current standards for HD water purity [34].

When micro-organisms are non-culturable, encapsulated in a biofilm or dead, the BC will remain negative but still bacterial derivatives, which are biologically active, might be present. To fill this gap, BCs need to be complemented with detection methods allowing quantification/indication of these bacterial fragments, as described below.

**Lipopolysaccharides**

LPS can be quantified by the limulus amoebocyte lysate (LAL) test. Different types of assay are either based on a gel-clot, turbidimetric, colorimetric or fluorescent principle. The latter test uses a recombinant form of factor C, the first component in the horseshoe crab clotting cascade. The kinetic chromogenic assay is the most sensitive test, detecting ET up to a concentration as low as 0.005 EU/mL. LPS catalyses the activation of a proenzyme in the lysate of the amoebocytes of the horseshoe crab, which catalyses the cleavage of yellow p-nitroaniline (pNA) from the colourless substrate Ac-Ile-Glu-Ala-Arg-pNA. The released pNA is measured photometrically, at 405 nm continuously throughout the incubation period. The ET concentration in a sample is calculated from the reaction time in comparison with the standard solutions. LPS fragments below 8000 Da are not detected by the LAL test, although even fragments with a molecular weight below 3000 Da, corresponding to the monomeric form of ET, still induce IL-1 [15]. Thus, the lack of LAL reactivity is insufficient evidence for the absence of biologically active LPS in HD fluids.

**Peptidoglycan**

PGs can be quantified with the silkworm larvae plasma (SLP) test, although this method is not routinely used and not recommended in the standards. SLP contains all the factors of the prophenoloxidase cascade, an insect self-defence mechanism. The cascade is initiated by PG, in which prophenol oxidase is ultimately activated to phenol oxidase. Phenol oxidase activity is colorimetrically detected, with 3,4-dihydroxyphenylalanine as a substrate and melanine as an end-product.

The test does not detect ETs, but cross-reacts with β-glucans, which produce a positive result in both the SLP and the LAL test. PG, on the other hand, is positive in the SLP test but negative in the LAL test.

**Bacterial DNA**

bDNA can be quantified using different methods. As described by Schindler et al. [22], the concentration of DNA extracted from dialysis fluid can be determined spectrophotometrically by measuring absorbance after exposure to ultraviolet light at 260 nm. Bossola et al. [25] described the detection of free bDNA by 16S rRNA gene polymerase chain reaction amplification after which the amplicons could also be used for identification purposes. Quantification of oligonucleotides (and ssDNA) can also be performed using OliGreen, an ultrasensitive fluorescent nucleic acid stain. In a microplate setting, as little as 1 ng oligonucleotide or ssDNA per millilitre can be detected (Quant-it®; Invitrogen). Considering the relevance of bDNA in HD, this parameter should be considered for routine use in future.

**Bioassay**

From the information above, it can be concluded that there are conditions (e.g. contamination by non-culturable micro-organisms or bacterial derivatives other than LPSs) where the detection of biologically relevant contaminants can be missed when applying the standard detection methods (BC and LAL test) recommended by the EBPG [30] and even the latest ISO 11663:2009. To overcome these limitations, non-specific but sensitive cell-based assays have been described, using whole blood, isolated peripheral blood mononuclear cells or monocytic cell lines. These assays are based on the cytokine (IL-1β or Tumor Necrosis Factor-α) inducing capacity of bacterial derivatives (cytokine induction assay, CIA). A bioassay using whole blood has been described by Lonnemann [35]. There was no correlation between the bacterial growth in dialysis fluid samples and cytokine induction capacity of the dialysis fluid sample in the whole blood assay. Previously, it had been demonstrated that there was also no correlation between bacterial growth and ET concentration measured by the LAL test [36]. To overcome
the need for healthy donors and the donor-related variability inherent to the whole blood assay, a calcitriol differentiated monocytic (THP-1) cell line can be used [17]. The assay is sensitive to 0.1 ng of intact LPS/mL (Escherichia coli 0111:B4) and LPS fragments <5 kDa (Pseudomonas aeruginosa), to 5 ng of PG/mL (Staphylococcus aureus) and to 0.1 μM oligodeoxynucleotides [2006 stimu (24 mer)] [17]. In the case of dialysis fluid, the test is considered positive if the induced cytokine (IL-1β) level is more than twice the background (BG). It is of note that the net pyrogenic activity of dialysis fluid may be best described by a cell-based CIA. Nevertheless, the response of the cell-based test system should and could be made more specific and sensitive. If so, these assays could be provided in a commercial kit making it more user-friendly for the routine setting. Adding a cell-based assay to the classical methods will be helpful in detecting the bacterial derivatives, which are currently missed with the classically recommended detection methods; this will contribute to the prevention of biofilm formation and could also contribute to decreasing micro-inflammation in HD patients.

How to implement quality control in your clinical practice and what will the estimated (material) cost be?

Depending on the type of HD therapy [low-flux HD (LFHD), high-flux HD (HFHD) or HDF], different standards are to be met (Table 1); this will have its implications on the mandatory sensitivity of a specific test.

First of all, technical or nursing staff should be trained to perform the sampling in a sterile and standardized way (cf. Figure 2).

For BC, if a microbiology laboratory is present in the centre or hospital, it can perform the BC, but only if they adapt the culture environment to the needs (nutrient poor medium, incubation temperature and time) described above. Depending on the standards needed, a pour plate (<100 CFU/mL, water and standard dialysis fluid) or filtration (<0.1 CFU/mL, ultrapure water or dialysis fluid) method should be used. The BCs can even be performed locally (in a separate room) by the technical staff, where sterile handling is assured, since prepared medium is commercially available and costs around €1/plate.

For the LAL test, as already mentioned, different tests are available with a sensitivity ranging between 0.005 and 0.01 EU/mL. Gel clot assays can be performed on-site since a calcitriol differentiated monocytic (THP-1) cell line can be used. The most sensitive kinetic chromogenic assay costs €23/sample.

For the bioassays, a laboratory equipped for cell culture handling is currently still mandatory but, when infrastructure is available, the assay is not too complex to introduce. In view of its undeniable additional value, offering an estimation of the in vivo relevant inflammatory capacity of dialysis water and/or fluid. Thus, adding a bioassay as described above to the classical detection methods is certainly worth considering. The cost is in the range of the LAL test.

There is no excuse not to perform the recommended tests of the quality of dialysis fluid, based on the classical detection methods (BC and LAL test). This can be considered as an insurance, preventing much larger costs when biofilm would develop necessitating the replacement of the complete water treatment system and/or distribution pipelines.

Approaches for action upon positive sampling results

The following part includes possible approaches for action in response to water and dialysis fluid purity results, obtained at monitoring and suggesting contamination. This approach is based on the procedure followed in our HD unit, where in general on-line HDF is performed. Our approach aims at a long-term preservation of the quality of the water treatment and distribution system, finally resulting in ultrapure dialysis fluid according to the standards recommended by the ISO 11663:2009 [31].

General disinfection procedure

In-centre HD. In the in-centre setting, the water distribution pipelines are heat disinfected (∼90°C) three times per week; this process is driven by a hot feed installed downstream the RO membrane. The HD machines are also included in the circuit covered by this heat disinfection procedure. In addition, a chemical disinfection of each HD machine is performed after each session, while after 100 treatments, the ultrafilters, used for ET and bacteria retention, are replaced followed by a chemical disinfection.

Disinfection of the RO membrane itself is only performed by the manufacturer, and only when the circuit has been opened for maintenance purposes or if, during monitoring, the alert levels or standard values of ET and/or BC are exceeded.

Although storage tanks should be avoided because of the risk of biofilm formation, it might be unavoidable to use them in some units. In which case, they can be subjected to UV irradiation, which is, however, only effective to kill planktonic micro-organisms. In addition, they should be frequently drained and adequately disinfected.

Home HD. In the home setting, the RO is chemically disinfected twice a month and a combination of chemical and heat disinfection of the HD machines is performed after each session. For the time being, we cannot apply
integrated heat disinfection (circuit and machine) in the home setting but aim at introducing it as soon as the implementation of this option becomes possible.

It is of note that the same quality as obtained in centre is also to be achieved at home. Patients need to follow an extensive training programme in the hospital before initiation of home dialysis including aspects of the preservation of dialysis water and fluid purity. The water treatment system is installed by the manufacturer, and the system is validated before use. The pipeline loop connecting the RO to the machine and vice versa should not be longer than necessary, but might vary in length depending on the location of the HD machine. The volume of disinfectant should be adapted accordingly. In addition, the pipeline coming from the RO should be connected directly to the machine, avoiding dead ends, with a sample port installed near the end of the return of the pipeline to the RO, avoiding opening of the system for sampling purposes.

Monitoring of the microbial quality

In-centre HD. Monitoring of the microbial quality of the water which is used to prepare the dialysis fluid is performed monthly on samples obtained behind the RO membrane and at the end of the pipeline. In addition, the microbial quality of the dialysis fluid is assessed twice a year. Sampling is performed at least 1 week after routine disinfection. When urgent resampling after an additional disinfection is needed (described below under ‘Interpretation of dialysis water samples’), a waiting period of at least 24 h should be respected, to avoid false negative results.

On each sample, an LAL test (ET quantification), a BC (R2A or TGEA), a fungus and yeast culture (SDCA medium, 22°C, 7 days) and a THP-1 assay (CIA) are performed.

Of note, when storage tanks are present, the water quality should be monitored on samples drawn from a port at the outlet of the water storage tank or at the first outlet to the distribution loop.

Home HD. Monitoring of the microbial quality of the water which is used to prepare the dialysis fluid is performed monthly on a sample obtained at the end of the pipeline. The microbial quality of the dialysis fluid (at the inlet of the dialyser) is assessed twice yearly.

General aspects concerning interpretation of water and dialysis fluid quality

The suggested approaches are depicted in flowcharts and are applicable to both in-centre and home HD.

Interpretation of dialysis water samples. In general, our flowcharts are based on the fact that the ET results become available first (~24 h after sampling) while the value is either (I) <0.250 EU/mL (Figure 3) or (II) ≥0.250 EU/mL (Figure 4).

When performing LFHD, HFHD and HDF, the total ET concentration and bacterial count in dialysis water should not exceed 0.250 EU/mL and 100 CFU/mL, respectively, according to the current recommendations.

The interventions when ET is <0.250 EU/mL (I) can, in view of the different action/alert levels, be subdivided into (Ia) the ET value below the alert level (<0.125 EU/mL)—the left side of Figure 3—and (Ib) the ET level

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Fig. 3. Interpretation of water sampling results, based on ET levels below 0.250 EU/mL. Flowchart when ET: < 0.250 EU/mL. Water sampling: monthly (i) behind the RO and (ii) at the end of the piping system. ET, endotoxin level by the LAL test; EU, endotoxin units; BC, bacterial culture; CFU, colony forming units.
above the alert level but below the standard level (0.125–0.250 EU/mL)—the right side of Figure 3.

When the ET level is <0.125 EU/mL (Ia), no direct action is to be undertaken but the results of the BC, which become available only after 7 days, should be awaited. When BC is also below the alert level (<50 CFU/mL), the routine screening procedure can be continued. When BC is within the alert level range (50–100 CFU/mL), an immediate disinfection of the RO and/or pipeline is performed, depending on the location in the circuit where the positive result was obtained. Subsequently, routine screening is resumed. When BC reaches or exceeds 100 CFU/mL, the procedure depicted under (II) ET ≥ 0.250 EU/mL and/or BC ≥100 CFU/mL (Figure 4) is followed.

When the ET level is above the alert level but still below the standard level (Ib): 0.125–0.250 EU/mL, an immediate disinfection is performed. When later on the BC value is <100 CFU/mL, the routine screening schedule is resumed, but from 100 CFU/mL on, again the sequence depicted in (II: Figure 4) is followed.

Figure 4 depicts the actions undertaken from (II) an ET value of 0.250 EU/mL on and/or a BC value of ≥100 CFU/mL. In this case, HDF and/or HFHD is stopped and switched to LFHD in all patients if machines are equipped with retentive filters and preferably with a dialyser containing a synthetic membrane. In addition, an immediate and thorough disinfection and/or cleansing of the RO and/or the whole circuit is performed. Next, dialysis water sampling is repeated and in addition dialysis fluid is collected from machines located at the beginning, middle and end of the pipeline to check whether the dialysis water contamination influences the quality of the dialysis fluid (procedure for dialysis fluid as depicted in Figure 5 and described below). If after thorough disinfection, ET and BC fall below the alert level, HDF or HFHD can be restarted and routine screening is resumed. On the other hand, a serious condition, with the suspicion of established biofilm, has developed if after thorough cleansing and disinfection values remain within the alert level range (ET: 0.125–0.250 EU/mL and/or BC: 50–100 CFU/mL). Original therapy (HDF or HFHD) can be resumed but additional measures such as the removal of biofilm should be considered. If levels remain at the standard level or above (ET ≥0.250 EU/mL and/or BC ≥100 CFU/mL), even the standard HD cannot be performed and more profound additional measures, such as the replacement of the RO and/or the pipeline, should be considered.

Interpretation of dialysis fluid samples from individual delivery systems. For HFHD and HDF, ultrapure dialysis fluid (ET: <0.03 EU/mL and BC: <0.1 CFU/mL) is required [32]. Where with LFHD, the current guidelines recommend that the total ET concentration and bacterial count in dialysis fluid should not exceed 0.50 EU/mL
However, to avoid the biofilm formation, ultrapure dialysis fluid (the same as with HDF and HFHD) is the appropriate solution. As depicted in Figure 5, from a dialysis fluid ET value of ≥0.03 EU/mL and/or a BC value of ≥0.1 CFU/mL on, the preferred action is to immediately remove the machine for cleansing and disinfection. This action is easy to implement in an in-centre setting where technicians are available and the replacement of machines is possible, without needlessly alarming the patient. However, when an immediate replacement of the dialysis machine is not possible, switching to LFHD can be a temporary alternative when the following conditions apply: the ET value between 0.03 and 0.5 EU/mL and/or the BC value between 0.1 and 100 CFU/mL (left side of Figure 5). In all other cases, reaching or exceeding the levels for the standard dialysis fluid (ET ≥0.5 CFU/mL and/or BC ≥100 CFU/mL), there is only one option which is to stop treatment (even LFHD) with this specific machine until it has been cleansed, disinfected and proven safe after resampling (right side of Figure 5).

If after cleansing and disinfection of the HD machine, the levels for ET and BC are below 0.03 EU/mL and 0.1 CFU/mL, respectively (ultrapure dialysis fluid), LFHD as well as HFHD or HDF can be restarted. If not, therapy should be adapted according to the results, as depicted in the upper part of Figure 5 and additional measures such as the removal of biofilm (the level is still at or above that requested for ultrapure dialysis fluid) or the replacement of the tubings and/or filters (the level is still at or above that requested for the standard dialysis fluid) as depicted in the lower right part of Figure 5 should be considered.

For the sake of completeness, the approach depicted in Figures 3–5 does not take into account a trend analysis of sample results over time which may be useful for the correct interpretation of the data; in that case, an evaluation of all results of the previous 3 months should be considered, allowing the identification of sampling errors which could be at the origin of unnecessary disinfection measures. In general, this trend analysis implies that (i) if the alert level for ET and/or BC is exceeded and based on the results of the previous months a sampling error is suspected (i.e. no trend), a resampling is requested; if the reported level is a confirmation of the previous increased levels, a disinfection is necessary; (ii) if the standard value for ET and/or BC is reached or exceeded and a sampling error is suspected, a resampling followed by an immediate disinfection, without waiting for the results of
the resampling, is performed. If the previously reported level exceeding the standard threshold is confirmed in the repeat sample, the HD strategy must be downgraded to LFHD (described above under ‘Interpretation of dialysis water samples’ in the last paragraph), followed by a thorough disinfection and a resampling to evaluate the efficiency of the disinfection procedure.

The depicted flowcharts also do not take into account the results of the CIA because they are not yet recommended by the national or international standards, but are in our hands considered simultaneously with the results of the BC. The induced cytokine expression is considered too high when exceeding three times the BG cytokine level for water samples and two times the BG for dialysis fluid samples. In case these thresholds are exceeded, the same measures are taken as for exceeded BC thresholds. The reader is welcome to contact us to obtain the extended procedure.

**Outcome of dialysis patients**

Up to now, the translation of disturbances of the quality of dialysis fluid into measures of clinical relevance has not always been satisfactory, mostly due to the fact that studies on which these approaches should be based, suffer from shortcomings such as lack of control group, a small patient sample or no reporting of dialysis fluid quality data. Although several studies have indicated that the use of ultrapure dialysis fluid improves inflammation-related parameters such as CRP, albumin or haemoglobin [37, 38], evidence of influence on hard end points such as cardiovascular morbidity and mortality is lacking. It will probably be very difficult to ever develop multicentric controlled trials comparing patients on ultrapure versus less pure dialysis fluid. However, starting from the knowledge that inflammation in dialysis patients, even with a CRP in the high normal range, increases the risk for cardiodependent trials comparing patients on ultrapure versus

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**Conclusion**

A combination of an adequately designed water treatment and distribution system and regular monitoring of the levels of microbial contaminants in water and dialysis fluid followed by consequent and well planned actions when needed are of major importance for the long-term preservation of water and dialysis fluid quality according to the latest standards. Nevertheless, one should be aware that even the most recently recommended methods do not guarantee the detection of all possible contaminants present, possibly resulting in an underestimation of their inflammatory capacity. The addition of a cell-based assay would be a useful tool in evaluating the net pyrogenic activity of dialysis water and fluid and in detecting more than ET alone.

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**References**

What can the dialysis physician learn from kinetic modelling beyond Kt/V\text{urea}?

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The importance of dialysis kinetic modelling

Although patients with renal failure retain a large variety of solutes, Kt/V\text{urea} is the most widely applied marker for the adequacy of dialysis. The popularity of Kt/V\text{urea} is probably related to its simple calculation from pre- and post-dialysis urea concentrations avoiding formal kinetic modelling [1, 2], and on the ease with which urea concentrations can be determined in most laboratories. Using this approach, the calculation is based on simple equations which provide corrections for urea generation, volume contraction, convective clearance and compartment effects.

While insufficient urea removal has been related to morbidity and mortality of dialysis patients in several uncontrolled studies [3–5], two controlled studies showed that increasing dialysis dose, defined as a higher Kt/V\text{urea} did not improve survival [6, 7]. Hence, it might be considered that other factors play an equal, if not more important, role in the pathophysiological deterioration of patients with renal dysfunction, and that the focus on urea kinetics alone is not representative for the evolution during dialysis of most other uraemic solutes. Evidence of the biochemical impact of urea at concentrations found in uraemia is scanty. In addition, cell membranes are highly...