

# Bacterial DNA in Water and Dialysate: Detection and Significance for Patient Outcomes

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## Key Words

Hemodialysis · DNA · Dialysate · Inflammation · Bacteria

## Abstract

The fluid used for hemodialysis may contain DNA fragments from bacteria, which could be harmful for patient outcomes. DNA fragments from bacteria, containing the nonmethylated CpG motif, can trigger inflammation through the monocyte and lymphocyte Toll-like receptor 9, and these DNA fragments have been observed in dialysate. The fragments may transfer across the dialyzer into the patient's bloodstream during hemodialysis treatment. During hemodiafiltration, the fragments would be introduced directly into the bloodstream. The DNA fragments may arise from biofilm in the pipes of the water system, from growth of bacteria in the water, or as contaminants in the bicarbonate and salt mixture used for preparation of dialysate. Current filtration methods, such as Diasafe filters, are not able to remove these fragments. It would be prudent to seek to reduce or eliminate these contaminants. However, the cost and effort of decreasing bacterial DNA content may ultimately require substantial facility improvements; we therefore need to fund research studies to determine if modifications to reduce bacterial DNA content are clinically warranted. This research will require methods to accurately determine the species of bacteria that contribute the DNA, since this information will allow the source to be established as biofilm, bicarbonate mixtures, or other problems in the dialysis system such as bacterial growth or leakage during water preparation. In this

review, the evidence for bacterial DNA fragments will be examined and suggestions for further studies will be described.

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## Introduction

The fluid used for hemodialysis may contain DNA fragments from bacteria. There are several reasons for concern about such contamination.

DNA fragments from bacteria, containing the nonmethylated CpG motif, can trigger inflammation through the Toll-like receptor 9 on lymphocytes. Dialysate samples used in hemodialysis clinics have been demonstrated to be contaminated with some degree of these short DNA fragments from bacteria [1, 4] from various species that contain these nonmethylated CpG motifs.

The size of these fragments, 15–20 bp long (molecular weight about 10,000 Da), would in theory allow their transfer across the pores used for modern high-flux dialysis membranes, and into the patient's bloodstream. During hemodialysis, some degree of backfiltration has been reported [1]. During hemodiafiltration treatment, the fragments would directly enter the patient's bloodstream as dialysate is infused.

The DNA fragments may arise from biofilm in the pipes of the water system, from growth of bacteria in the water, or as contaminants in the bicarbonate and salt mixture used for preparation of dialysate. The fragments would not

be removed by the Diasafe filters now employed to reduce endotoxin content of the dialysate. The technical efforts to remove or reduce the bacterial DNA fragments might be complicated, and require substantial effort and expense.

For all these reasons, there is a theoretical justification for concern about inflammatory effects of DNA fragments present in dialysate. It would be prudent to seek to reduce or eliminate these contaminants. However, decreasing bacterial DNA content of dialysate may ultimately require a substantial investment in facility improvements; we therefore need to carry out research studies to determine if modifications to reduce bacterial DNA content are clinically warranted.

There is at this time no direct evidence that reduction of bacterial DNA fragments would benefit patients, but there is sufficient indirect evidence to pursue the hypothesis. In the remainder of this review, this evidence will be examined in detail, and suggestions for further studies described concurrently.

## Background

Purity of dialysate has been a technical challenge since the beginnings of dialysis treatment. To achieve the most efficient dialysis, the permeability of the dialyzer membrane has been progressively increased, and some high-flux dialyzers are permeable to molecules as large as 40,000 Da. The permeability is bidirectional, with transfer possible in both directions. Backfiltration is both diffusive and convective, and there may be 3–6 liters per treatment of reverse water movement in current high-flux dialysis treatment [5–7]. The use of hemodiafiltration [8], with deliberate introduction of 10–20 liters of dialysate per treatment [9] into the patient's bloodstream, introduces yet greater requirements into the need for high dialysate purity [10]. The shift to hemodiafiltration may lead to further technical advances in the preparation of high-purity fluids for dialysis treatment.

To achieve these goals, modern dialysis water systems use reverse osmosis and charcoal filtration to remove microorganisms, aluminum, chloramines, and other contaminants. A Diasafe filter can be used to remove high-molecular-weight endotoxin components [11, 12]. For example, switching from a deionizer to a reverse osmosis system led to decreased patient C-reactive protein levels [13]. Systems are periodically disinfected and flushed with chemicals in an effort to remove biofilm [14, 15]. All these efforts have led to ongoing improvements in the quality of dialysis water, which has become a major focus of dialysis

clinic management [15, 16]. The conversion to bicarbonate buffer increases the technical challenge, since bicarbonate solutions are better support media than acetate solutions for the growth of bacteria [17]. The primary improvements from 1990 to 2005 addressed modifications to decrease the quantity of viable bacteria and endotoxin in dialysate, especially because high-flux dialyzers may introduce pyrogens into the patient's bloodstream during treatment [18–20]. The benefits of switching to ultrapure dialysate were not observed when patients were dialyzed with low-flux dialyzers [21], indicating that the use of high-flux membranes has greater potential to transfer harmful constituents to the bloodstream.

These ongoing changes in preparation of dialysate are dictated by recognized hazards; when a harmful component is recognized in the water system, engineering changes are made to remove that component. This strategy cannot work for hazards that have not been identified, since special modifications have to be usually made for each harmful component. Much of the current emphasis on dialysate purity (ca. 2005) has shifted to bacterial DNA constituents: are they present? At what level? Are there sufficient amounts to pose a hazard during regular hemodialysis treatment, or during hemodiafiltration (HDF)? If the hazard is established, technical changes in dialysate preparation, dialyzer design, or other features of treatment are likely to be implemented to reduce this source of hazard.

## Bacterial DNA Motifs as Proinflammatory Elements

The DNA of humans typically contains a methylcytosine when there is a cytosine followed by a guanine (methyl-CpG). Cytosine methylation is an evolutionary mechanism used by vertebrates to achieve gene silencing, and since most genes in any cell are dormant, the majority of cytosines are methylated. It has been reported that 70–80% of all CpG sequences in the human genome are methylated at cytosine [22]. By contrast, the CpG sequence in bacteria is usually not methylated. The lack of methylation at bacterial CpG motifs has enabled the evolution of a monocyte and lymphocyte recognition system that responds to bacterial DNA, thereby enhancing innate immunity.

Nonmethylated CpG motifs in bacterial DNA fragments (15–25 bp in length) can trigger leukocyte activation through action on the Toll-like receptor 9. These changes include: increased cytokine release [1], prolonged mononuclear cell survival in culture [2], antibody class switching in B cells [23], NF- $\kappa$ B activation [24], TNF- $\alpha$

**Table 1.** Studies that have examined proinflammatory effects of measured amounts of bacterial DNA

Reference	Model used	Method of addition of DNA	Threshold level for activity	Outcome reported
He et al. [23]	Human germline B cells in culture	Added to cells in culture	5 µg/ml	Antibody class switching (from IgM to IgG)
Luyer et al. [26]	Induction of inflammation during hemorrhagic shock	Injected into the rat peritoneal cavity	5 µg/ml	Increased IFN-γ from isolated peritoneal macrophages
Navarro et al. [2]	Human mononuclear cells in culture	Added to cells in culture	1.8 µg/ml	Increased IL-1β expression, inhibition of cell apoptosis
Schindler et al. [1]	Human mononuclear cells in culture	Added to cells in culture	0.5 µg/ml	Increased production of IL-6
Chuang et al. [24]	HEK239 cells in culture	Added to cells in culture	5 µg/ml	NF-κB activation
Gao et al. [25]	RAW 264.7 macrophages in culture	Added to cells in culture, along with lipopolysaccharide	10 µg/ml	Increased TNF-α production

generation [25] and induction of IFN-γ by isolated peritoneal macrophages [26].

These studies are summarized in table 1, along with an estimate of the threshold level of bacterial DNA needed to bring about proinflammatory changes.

### Levels of Bacterial DNA in Water That Would Be Critical

Current PCR amplification protocols can be applied to find traces of DNA at levels below the threshold of inflammatory activation. Decisions on water system changes would include estimates of levels, as well as presence established by PCR.

From the activation thresholds in table 1, can we estimate the concentration in dialysate that might trigger inflammation? Five liters of dialysate water is a typical value for backfiltration during high-flux dialysis [5]. Assume 100% convective transport of short DNA fragments during backfiltration. Two hundred liters per treatment is a typical total dialysate flow, with 10% sieving from diffusive transport for molecules in the 10,000-Da size range. Assume the fragments in 20 liters of water enter because of diffusive transport. This means that the DNA contained in 25 liters of dialysate enters the bloodstream. The extracellular volume of distribution in a human is about 20 liters (including plasma and interstitial water).

For example, if the dialysate contains 1 mg of bacterial DNA per liter, then the 25 mg of DNA that cross into the bloodstream would be distributed in 20 liters of human tissue, at a concentration of about 1 µg/ml. This level of DNA in dialysate would lead to a proinflammatory concentration of bacterial DNA in tissues, based on the lowest threshold reported in published studies of 0.5 µg/ml (table 1). However, if the DNA concentration in the dialysate is 0.1 mg/l, the current model studies do not support the hypothesis that this level of DNA would be of concern. The value of bacterial DNA reported by Schindler et al. [1], 0.24 mg/l of dialysate, is near the threshold concentration of bacterial DNA that might trigger inflammation during a dialysis treatment. DNA levels may be much higher in other dialysate samples, and measurement of these levels is a key research objective.

### Bacterial DNA Transfer during Hemodiafiltration

During HDF, dialysate fluid is added directly to the external blood circuit, to increase the total amount of ultrafiltration achieved during the dialysis treatment. During HDF, the amount of bacterial DNA fragments that enter the bloodstream is directly proportional to the dialysate DNA concentration and the total HDF volume, which often reaches 20 liters or higher. For HDF treatment, the addition of bacterial DNA to the bloodstream

can be accurately calculated, in addition to the contribution from diffusive transfer, which is determined from modelling assumptions. The increasing use of HDF indicates that water purity, and potential effects of bacterial DNA, need to be critically evaluated with application of this technology to improve dialysis efficiency.

### **Tracing the Origins of Bacterial DNA in Dialysis Water**

The microbial flora that might contaminate dialysate water is complex: at least 200 different species have been cultured [27, 28], and there are additional organisms present that are not readily cultured using standard laboratory media. Typing bacterial species or genera with a 20-bp sequence is possible if a unique sequence has been obtained from PCR, but some sequences are common across many species.

Using a 15-mer, comparing the genomes of *Escherichia coli* O157H7 and *Pseudomonas aeruginosa*, and allowing for a single-base-pair substitution, there is a 16% probability that a given 15-mer will be present in some random sequence in both genomes. With a 20-mer, the probability is only 0.6%, and with a 25-mer, only 0.1%. If PCR amplification is done with 3–4 sequences, 20 bp or longer, definitive establishment of species origin would be achieved. This should be followed by quantitative PCR amplification to determine the relative contribution of that species DNA to the total bacterial DNA content.

Navarro et al. [2] and Schindler et al. [1] used PCR amplification of DNAb, a common motif found in bacterial 16S transfer RNA. Other species-specific motifs will need to be exploited to determine the species of bacteria that contribute the DNA fragments.

### **Quantitation of Bacterial DNA Present in Dialysis Water**

Highly sensitive PCR amplification will allow bacterial DNA fragments to be detected, even if those levels are lower than the threshold for harmful patient outcomes. Since very low levels of bacterial DNA fragments may be innocuous, there is the requirement for quantitation of DNA content to determine if the amounts of DNA present are potentially harmful to the patient.

Measurement strategies commonly rely on some method of enrichment, such as isolation of DNA from large quantities of water on a C18 cartridge [1]. After the

DNA has been isolated, it can be measured either by its OD260/OD280 ratio, by HPLC analysis of the digested DNA, or by quantitative PCR amplification of selected motifs [29]. Reliable methods for quantitation of bacterial DNA will be essential to make decisions on the needed modifications in dialysis water systems.

### **DNA Transfer across the Dialyzer Membrane**

Schindler et al. [1] reported the back transfer of bacterial DNA using an in vitro dialysis model; the absolute amount that crossed over to the bloodstream side was not reported. Further evidence of DNA transfer has recently been reported by De Cal et al. [30]. The importance of DNA contamination in dialysate will require the determination of the transfer coefficients of bacterial DNA fragments, and this core question must be answered by ongoing research.

### **Strategies for Determining the Inflammation-Inducing Properties of Dialysate**

Since the objective of this effort is production of dialysate that does not trigger inflammation, a direct assay of dialysate with cultured cells in vitro can be used to determine if that goal has been achieved. This has been described by Glorieux et al. [31], who incubated complete dialysate mixture with THP-1 cells and examined it for the induction of IL-1 $\beta$ . Among 270 samples of dialysate obtained from dialysis units, there were 21 dialysate specimens described as ‘high purity’ that gave a positive test result. The nature of the active contaminants was not characterized, and may have been endotoxin, endotoxin fragments, peptidoglycans, DNA fragments or unknown proinflammatory molecules. This type of in vitro assay can be used to monitor the success of routine water system maintenance procedures and new technical innovations that are carried out to minimize the presence of proinflammatory substances in dialysate.

### **Benefits from Decreasing the Content of Bacteria, Endotoxin, or Other Microbial Components in Dialysate**

It was established in the 1990s that endotoxin permeability was important with high-flux dialysis, especially because of reverse flow that occurs with high-flux proce-

dures. The direct introduction of dialysate into the patient's bloodstream that occurs with hemodiafiltration made dialysate purity a very high priority. These benefits are best established during conversion of the water system for an individual dialysis unit. Longitudinal measurements of C-reactive protein and erythropoietin dose provide excellent feedback on the decrease in patients' inflammatory status.

Benefits from improved water quality have been reported in several studies, where endotoxin and bacterial content were systemically reduced [32–34]. These studies showed decreased C-reactive protein, cytokine levels and erythropoietin requirements, and increased plasma albumin levels. Removal of bacterial DNA from ultrapure dialysate may require additional technical innovations; the benefits will need to be examined in trials that examine clinical indicators after reduction of bacterial DNA fragments in dialysate.

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