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Endotoxin Level Measurement in Hemodialysis Biofilm Using "The Whole Blood Assay"

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Abstract: Biofilms have been found on the inner surface of silicone tubing inside dialysis machines. Endotoxin releasing from those biofilms increases the bioincompatibility of dialysis liquids and leads to long-term inflammatory complications among dialysis patients. Endotoxin measurement is recommended for the control of dialysis liquids. This article describes the use of a new method, the Whole Blood Assay (WBA), for endotoxin quantification in dialysis biofilms. Biofilms were suspended in sterile water by scraping the tubing samples. Diluted blood samples from healthy donors were stimulated overnight with the contaminated suspension. Stimulated mononuclear cells released IL-1β in response to endotoxins. IL-1β level was then measured using an ultrasensitive ELISA method. We demonstrated a semi-logarithmic model in which the optical densities measured after the ELISA assay increases linearly with the levels of endotoxin. This model allowed the determination of the amount of endotoxins in biofilm samples with a detection limit of 0.032 EU/mL. Most of the time, the amounts of endotoxin measured by the WBA were higher than those measured by the Limulus Amoebocyte Lysate (LAL) assay. This study suggested the presence of "endotoxin-like" compounds different from the lipopolysaccharides that are not detected by the LAL assay. We concluded that the LAL is necessary but insufficient to have a representative quantification of endotoxins that could be hazardous to patient health. **Key Words:** Endotoxins—Biofilm—Hemodialysis—IL-1β—Human mononuclear cells.

The human immune system is known to respond to bacterial components, the best example being lipopolysaccharides (LPS) of the outer membrane of gram-negative bacteria, also called endotoxins (1). Numerous studies have demonstrated that endotoxins have the potential to induce an inflammatory response by stimulating circulating monocytes that release cytokines like IL-1β, IL-6, TNF-α, and cause significant health problems even at low levels (2). Recently, many authors have found that most bacterial compounds, such as exotoxins, lipoteichoic acids and peptidoglycans of gram-positive bacteria, even bacterial DNA, have similar properties (3–5).

Health risks related to endotoxins are common when these compounds enter the patient's blood stream. Industrial companies are careful to produce endotoxin-free drugs or medical devices (such as needles and shunts) for therapeutic use, but problems frequently occur in systems such as hemodialysis machines that are difficult to sterilize and expose the patient to endotoxins. In most cases of hemodialysis, the dialysate (a mineral salt solution that contacts the blood of the patient through a semipermeable membrane) is prepared with large amounts of nonsterile purified water. Moreover, the level of endotoxins in the dialysate is increased by biofilm development in fluid pathways within hemodialysis systems, with the most significant formation occurring inside the silicone tubing of dialysis machines (6–8). Biofilms are adherent bacterial communities surrounded by an exopolymeric matrix that are commonly associated with persistent infection (9). Biofilms are highly resistant to antimicrobial treatment and have been shown to form significant colonization on minimal nutrients. Research studies have shown that biofilms are capable of releasing large quantities of endotoxins into hemodialysis liquids (Marion-Ferey et al., unpublished studies).

Endotoxins have been shown to cross the dialysis membrane and many inflammatory reactions have

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been associated with the practice of hemodialysis (10,11). Repetitive cytokine induction contributes to some of the long-term complications among dialysis patients. This chronic stimulation of the inflammatory system during hemodialysis is called bioincompatibility (12).

The concerns with bioincompatibility have led to the development and implementation of standards for the level of endotoxins and microbiological contamination in hemodialysis liquids. The European Pharmacopoeia does not allow more than 0.25 Endotoxin Units (EU)/mL in hemodialysis water (13). The AAMI (Association for the Advancement of Medical Instrumentation) sets the upper level of endotoxin concentration in the dialysis system to 2 EU/mL (14). However, many studies demonstrate the need to decrease these threshold values to obtain an ultrapure dialysate containing less than 0.1 Colony Forming Unit (CFU)/mL and 0.03 EU/mL (11). Such a low level of contaminant dialysate is needed for the on-line hemodiafiltration in which the dialysate goes through ultrafilters and is then injected into the patient's blood (15). The presence of standards and the increase of on-line hemodialfiltration practice involves regular control of the endotoxin levels in the dialysis water and dialysate. The conventional method recommended by the European Pharmacopoeia and the AAMI for those controls is the LAL (Limulus Amoebocyte Lysate) test (16). This test is limited to accurately detecting complete LPS molecules and lipid A, however, other "endotoxin-like" components escape detection (17,18). Additionally, nonspecific interactions complicate the analysis by giving false negative or positive readouts (19–21).

To overcome these limitations, a new method for the measurement of endotoxin levels in human blood has been developed. This method, called "Whole Blood Assay" (WBA) is based on the stimulation of the human mononuclear cells by the endotoxins, leading to synthesis and release of inflammatory cytokines such as IL-1β, IL-6, and TNF-α in the supernatant fluid, as measured by an ELISA assay. Through this technique, the level of cytokines is easily correlated to the concentration of total endotoxins in the sample. Previous studies have demonstrated the efficacy of this approach in other systems (1,22,23), It has also been recently applied to study dialysis systems and dialysate ultrafiltration for on-line hemodiafiltration practice (19–21).

This article discusses the use of this new method for quantifying endotoxins in biofilms in hemodialysis systems. The methodology has been modified from that used in Fennrich's studies. The modifications were performed in order to reach two objectives. The first was to use directly the relationship between the optical densities given by the ELISA assay and the endotoxin levels as a calibration curve; the second was to increase the sensitivity of the method using ultrasensitive ELISA kits, allowing the detection of trace amounts of endotoxins in thin hemodialysis biofilms. The effectiveness of this test was evaluated and then applied to the analyses of hemodialysis systems.

MATERIALS AND METHODS

Biofilm removal

The internal surface of silicone tubing samples covered by biofilms was scraped for 2 min using the mechanical biofilm scraper we previously described (24). Bacterial aggregates were dissociated by drawing the suspension three times through the needle of a syringe (22 G, 0.7 mm) followed by 1 min of vortexing. The endotoxin level was then determined on the resulting suspension and reported to the surface area of sample. Knowing that the tubing samples sizes were all 2 cm in length and 4 mm ID (internal diameter), the surface area of each sample was 2.5 cm^2.

Mononuclear cell stimulation

Blood samples were drawn from healthy donors. Individuals with inflammatory disorders and/or undergoing treatment for inflammation were excluded. Blood samples were stored at 37°C for at least 4 h before analysis. Endotoxin standard samples or bacterial suspension samples (resulting from biofilm scraping) were serially diluted with sterile endotoxin-free water (LAL water, Charles River Endosafe, Charleston, SC, U.S.A.) into sterile and endotoxin-free microcentrifugation vials (Safe-lock Eppendorf Biopur, Fisher Scientific, Pittsburgh, PA, U.S.A.) and agitated according to the LAL assay protocol (16). Standard endotoxin solution (RSE, *E. coli* 055B5, 160 EU/mL (10 ng/mL), Charles River Endosafe) was serially diluted in order to obtain 10 standard solutions ranging from 0.032 to 8 EU/mL (0.002–0.5 ng/mL). In sterile endotoxin-free 24 well plates (Falcon 35 3047, Fischer Scientific), 200 µL of whole blood was diluted with 1 mL of NaCl 0.9% (Baxter, Maurepas, France) and 100 µL of standard or bacterial sample were added. For each analysis, three negative controls were prepared replacing the standard by 100 µL of sterile endotoxin-free water. The 24-well plate was then incubated overnight at 37°C with 10% CO_2 (CO_2 incubator, model 3110, Forma Scientific Inc, Marietta, OH, U.S.A.). During

incubation time, the mononuclear cells (MNC) released IL-1β into the supernatant in response to the endotoxins.

IL-1β level measurement

After incubation, 100 µL of supernatant was taken from the plate and was added to a 96-well plate of an Ultra-Sensitive IL-1β ELISA kit (Cytoscreen, Biosource International, Camarillo, CA, U.S.A.) without previous centrifugation. The ELISA assay was performed per manufacturer's instructions at room temperature. Each sample was analyzed in duplicate. The final optical densities (OD) were measured in each well by a microplate reader (model EL 808, Bio-Tek Instruments, Winooski, VT, U.S.A.), at room temperature at 450 nm. Data were analyzed using K4C (Bio-Tek Instruments) and Excel (Microsoft) software. Results were given in EU/mL for liquid samples and EU/cm^2 for biofilm samples. The duration time of the ELISA assay was 3 h.

Analytical evaluation of the technique

The method was evaluated according to the recommendations described by the European Agency for the Evaluation of Medicinal Products (EAEMP) (25).

Limulus Amoebocyte Lysate (LAL) assay

Endotoxin levels in the bacterial suspension samples were determined using the LAL chromogenic kinetic assay (Endochrome-K Kits, Charles River Endosafe). The standard procedure described in the European Pharmacopoeia text #2614 addendum 2001 was used on 100 µL of samples (16). Kinetic analyses were performed at 37°C with the microplate reader with a time to onset OD of 0.100. The LPS from *E. coli* 055B5 (Charles River Endosafe) was used to calibrate the method.

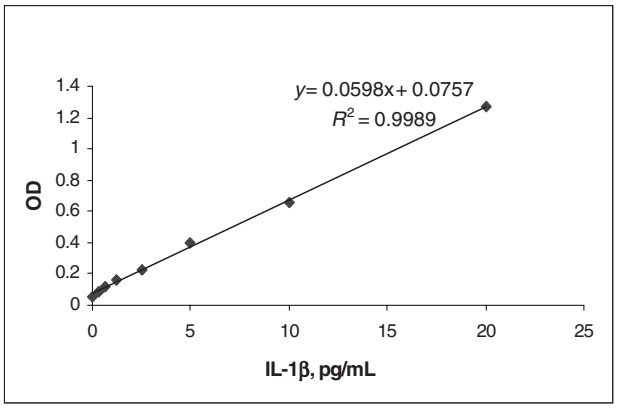

FIG. 1. IL-1β calibration curve obtained with the ELISA IL-1β US kit.

TABLE 1. *Basic level of IL-1β from six donors*

Donor #	Average of the duplicates	Intradonor average	SD	VC%
1	0.70 0.45 0.52	0.56	0.13	23.2
2	0.26 0.34 0.50	0.37	0.12	32.4
3	0.41 0.70 1.08	0.73	0.34	53.4
4	0.86 0.39 0.42	0.56	0.26	46.4
5	0.86 0.54 0.79	0.73	0.17	23.3
6	0.61 0.31 0.63	0.52	0.18	34.4
Interdonor variability		0.58	0.086	14.8

Each sample was treated in triplicate and each triplicate was analyzed in duplicate by the ELISA assay. Values are given in pg/mL.

RESULTS

Evaluation of the ELISA method

The quantification of the cytokines released by the mononuclear cells (MNCs) was performed with an ELISA kit according to a calibration curve that gives the relationship between the OD and the levels of IL-1β in the standards. For each analysis, the linearity of the calibration curve validates the ELISA assay. Figure 1 shows a linear calibration curve ($R^2 = 0.9989$).

Inter- and intradonor variability

Inter- and intradonor variability was determined by measuring the basic blood level of IL-1β (blanks) with six different donors before stimulation with the endotoxins. For each donor, three tests were performed for one donation and each sample was analyzed by ELISA in duplicate. Table 1 gives the averages for each duplicate ($n = 2$), the averages, standard deviation, and variation coefficients for each donor ($n = 6$), and for the 6 donors ($n = 36$). Values are given in pg/mL. Results show a very low interdonor variability (14.8%). The highest values of the intradonor variation coefficients may be explained by the limited number of measurements ($n = 6$). However, most of those values could be considered as normal, when comparing biological samples.

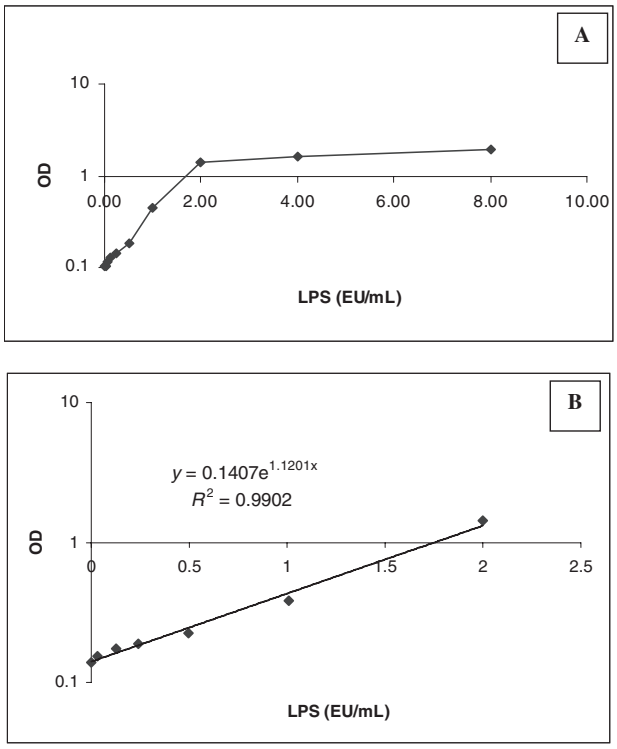

FIG. 2. (A) Relationship between the levels of LPS in the standards (0–8 EU/mL) and the OD measured after the ELISA assay (semilogarithmic scale). (B) Exponential equation for LPS levels ranging from 0 to 2 EU/mL.

Evaluation of the WBA

Figure 2A shows the relationship between the levels of LPS in the standards ranging from 0 to 8 EU/mL and the OD measured after the ELISA assay (semilogarithmic scale). Note that the relationship is linear for the seven lowest LPS levels (ranging from 0 to 2 EU/mL). The exponential equation obtained shows a slope of 1.120 (coefficient of the equation), an intercept of 0.14 and a linearity coefficient $R^2 = 0.990$ (Fig. 2B).

Above 2 EU/mL, the relationship reaches a saturation range. For this reason, samples with endotoxin concentrations larger than 2 EU/mL will have to be diluted.

Six calibration curves were prepared to determine the reproducibility of the method, with six different donors. The averages of the OD measured for each standard, the averages of the slopes for each curve, the standard deviations, and the variation coefficients (VCs) are given in Table 2.

The VCs are around 25% for each standard. Knowing that this method is a biological method, this value can be considered to have a good reproducibility. Moreover, the reproducibility of the slopes and R^2 are in acceptable ranges (VC = respectively 11.529 and 0.208).

The accuracy of the method was then determined by calculating the percentages of deviation between the theoretical and the calculated concentrations of each standard of each calibration curve (% dev = (theoretical conc – calculated conc)/theoretical conc). Results are shown in Table 3. For concentrations ranging from 0.240 to 2.000 EU/mL, the absolute values of the percentages of deviation are all below 31%, meaning that the method is quite accurate. But these percentages of deviation increase rapidly for the lowest concentrations (0.032 and 0.128 EU/mL), except for the calibration curve #1. As a comparison, the accuracy of the LAL assay was determined on six different calibration curves according to the same calculations. Results are given in Table 4. Two calibration curves show a very high percentage of deviation for the two lowest concentrations (from 0.005 to 0.05 EU/mL).

Finally, the detection limit was determined and defined as the lowest concentration of standard that

TABLE 2. *Reproducibility and linearity of the WBA. Validation with six calibration curves (CC)*

Theoretical concentration (EU/mL)	CC1 (OD)	CC2 (OD)	CC3 (OD)	CC4 (OD)	CC5 (OD)	CC6 (OD)	Average ($n = 6$)	SD	VC percentage
0.000	0.119	0.102	0.102	0.105	0.176	0.139	0.124	0.029	23.634
0.032	0.133	0.105	0.112	0.125	0.209	0.154	0.140	0.038	27.244
0.128	0.154	0.127	0.131	0.171	0.245	0.174	0.167	0.043	25.705
0.240	0.182	0.145	0.152	0.180	0.276	0.189	0.187	0.047	25.023
0.496	0.215	0.185	0.170	0.247	0.354	0.225	0.233	0.066	28.182
1.008	0.376	0.447	0.460	0.521	0.577	0.382	0.461	0.078	17.021
2.000	1.270	1.420	1.600	2.380	2.199	1.430	1.717	0.460	26.773
4.000	*1.305*	*1.638*	*1.867*	*2.567*	*2.314*	*1.611*	*1.884*	*0.474*	*25.148*
8.000	*1.418*	*1.946*	*2.106*	*2.620*	*2.421*	*1.876*	*2.064*	*0.425*	*20.607*
Slopes	1.136	1.331	1.371	1.486	1.189	1.120	1.272	0.147	11.529
R^2	0.995	0.995	0.991	0.993	0.992	0.990	0.996	0.002	0.208

The values for concentrations 4 and 8 EU/mL are indicated in italics to show the saturation effect in all samples but are not included in the calculation of slopes and R^2.

TABLE 3. *Accuracy of the WBA*

Theoretical concentration (EU/mL)	Percentages of deviation					
	CC1	CC2	CC3	CC4	CC5	CC6
0.032	–26.9	47.1	–68.9	14.1	–82.2	–165.9
0.128	–32.5	–44.0	–31.5	–86.2	–50.0	–51.6
0.240	–31.9	–25.4	–15.3	–13.7	–21.7	–11.6
0.496	6.6	–3.9	27.7	2.0	–1.1	14.6
1.008	5.2	–28.2	–7.6	2.0	9.5	11.1
2.000	–1.3	–15.5	0.3	–0.5	–1.9	–3.7

gives a signal different from the blank. The detection limit of our method was 0.032 EU/mL (0.002 ng/mL).

Application to hemodialysis samples

The WBA method was applied to various hemodialysis samples that were: one hemodialysis water sample, one dialysate sample, and 15 tubing samples after biofilm removal. All the samples were also analyzed using the LAL method (24) in parallel to the WBA method described here. Results are given in Table 5. No aberrant values were obtained demonstrating that this assay worked well for analyzing biofilm samples and that removal of the biofilm did not interfere with the signals. Interestingly, most values obtained with the WBA test were higher than those obtained by the LAL assay.

The endotoxin levels measured by the WBA test were more than 20 times higher than the corresponding level in the LAL assay. Only 5 samples out of 17 gave lower values with the WBA than with the LAL and the differences were from 7.8 to 38%. However, the correlation coefficient calculated with the LAL and WBA values from the 17 samples was $R^2 = 0.964$, showing a very good correlation between those two methods.

DISCUSSION

This article describes a novel method for endotoxin-level measurement adapted for the biofilm risk assessment in hemodialysis systems. Health risks related to hemodialysis are mostly due to endotoxins. Thus, a good analytical method for endotoxin measurement is needed for the control of the microbiological quality of hemodialysis liquids. Moreover, the ability to follow biofilm development on the inner surface of silicone tubing inside dialysis machines is necessary for the control of dialysis systems and endotoxin measurement is one of the most important parameters that needs to be taken into account for biofilm quantification and the effects of biofilms on patient health.

TABLE 4. *Accuracy of the LAL assay*

Theoretical concentration (EU/mL)	Percentages of deviation					
	CC1	CC2	CC3	CC4	CC5	CC6
0.005	4.0	0.0	–154.0	38.0	–20.0	–96.0
0.050	–25.2	17.8	72.8	–42.0	18.8	63.9
0.500	30.7	–39.9	–1.4	–47.3	–3.4	88.7
5.0	–30.2	6.7	–17.7	–14.7	19.2	–11.7
50.0	6.9	6.5	–21.6	31.9	–21.6	–11.7

TABLE 5. *Application to dialysis samples. Comparison with the LAL assay*

Samples	WBA*	LAL*
Hemodialysis water	0.22	0.28
Dialysate	0.44	0.20
Tubing sample (TS) #1	214 400	232 515
TS # 2	35 040	12 026
TS # 3	14 080	17 209
TS # 4	207 967	57 664
TS # 5	221 163	72 900
TS # 6	4024	2228
TS # 7	7.76	11.37
TS # 8	100	4.7
TS # 9	28 891	24 796
TS # 10	7824	1104
TS # 11	192	312
TS # 12	1 075 000	858 545
TS # 13	0.10	<0.005
TS # 14	0.20	0.01
TS # 15	3.67	0.40

*Units: Hemodialysis water and dialysate—EU/mL; Scraped tubing samples—EU/cm^2 (the surface area of each tubing sample is 2.5 cm^2).

The LAL assay has been used for more than 20 years for the control of hemodialysis liquids and drugs for injection and has become the standardized method for endotoxin detection. Since the presence of endotoxins in hemodialysis systems is still a large problem in the medical community, we found it necessary to consider another technique that could give a more complete quantitative measurement of total pyrogenic compounds, including "endotoxin-like" components as well as elucidate the reaction of the human immune system to endotoxinic stimulation.

The use of human monocytes as models to detect inflammatory reactions due to endotoxin was first suggested more than a decade ago. Some authors proposed isolating large numbers of monocytes from the peripheral blood of humans for these assays (17,18). Nevertheless, the complicated protocol lead other authors to examine the efficacy of employing monocytoid cell lines such as U937, THP-1, and HL-60 to study these interactions (26,27). Previously, we conducted studies on U937 cell lines and concluded that the first step of the method, the stimulation of the MNC by cell activators (PMA for example), was

not statistically reproducible (data not shown). Moreover, cell culture requires trained technicians and is not commonly used for routine analysis.

Thus, the method described here has many advantages. Our experiments required low volumes of blood (200 μL per sample), pretreatment was unnecessary, and whole blood more closely mimics the interactions observed in vivo (12).

To improve on the method of Fennrich (1) we wanted to be able to quantify the endotoxin level using a calibration curve that accounts for the relationship between the LPS level of the standards and the final signal, i.e., the OD measured after the ELISA assay. Importantly, other studies allow for the quantification of the level of cytokine without considering the stimulation rate by the endotoxins (1,19,20,21,22,23).

Pool first proposed a similar linear relationship that could be used as a calibration curve between the LPS levels and the OD, but the curve was made of only three points and the LPS concentrations used were too high to be of clinical significance (28). Our data support the development of this technique and demonstrate a semilogarithmic model in which the OD increases linearly with the levels of LPS from *E. coli* O55B5 ranging from 0.032 EU/mL (0.002 ng/mL) to 2 EU/mL (0.125 ng/mL).

Another goal of this work was to increase the sensitivity of the method similar to that of the LAL chromogenic kinetic assay, allowing the quantification of trace amounts of endotoxins. Some previous studies had reported a quantification limit of approximately 0.16 EU/mL (0.01 ng/mL) of LPS. The use of the ultrasensitive ELISA kits decreased this limit to 0.032 EU/mL. However, the background noise also increased with the ultrasensitive ELISA kits but still did not interfere with the positive signals and overall sensitivity. In some cases, the higher background could be traced to the glassware, as glassware heated to 180°C for 4 h still contained measurable levels of endotoxins. This observation could be reversed by employing single-use endotoxin-free assay plates. Most percentages of deviation comparing the calculated and theoretical concentrations were quite high but such levels can be related to the biological nature of the assay and the number of different steps of the techniques. However, this method seems not to be accurate enough to obtain relevant data for the measurement of traces of endotoxins. The LAL assay remains the most accurate method.

This study is in accordance with previous observations about the presence of "endotoxin-like" compounds that are not detected by the LAL assay. The majority of liquid and biofilm samples had a level of endotoxins measured by the WBA higher than the level of endotoxin measured by the LAL assay. Similar results have been obtained by other authors (17,18). This study suggests that the LAL assay is necessary but not sufficient to have a representative quantification of the amount of total pyrogenic compounds that could be hazardous to patient health. The WBA seems to be a much better test of overall contamination of hemodialysis samples.

This article describes a new approach to endotoxin analysis in biofilms but further work is required before this method is considered to be validated. As a matter of fact, these first results have to be completed by the analysis of a large number of clinical samples and by statistical studies for both the assessment of reproducibility and the comparisons between the endotoxin levels measured by the LAL and WBA assays.

The two main disadvantages of this new method are the price of the ELISA kits (around $380 for 96 tests) and the difficulties of obtaining fresh blood samples. In comparison with the current standard LAL analysis, the WBA assay has a cost increase of about 40%. Up to now, methods for endotoxin quantification are expensive. The LAL assay involves horseshoe crab breeding and lysate purification. The WBA uses expensive ultrasensitive ELISA kits. However, a routine application of this assay should lead to a decrease in the cost.

Our experiments were performed with blood provided by healthy donors. A routine application of the assay will require an agreement between the laboratory and a blood bank to obtain large amounts of fresh blood. This approach represents a complicated step that increases the difficulties of routine application.

CONCLUSION

The Whole Blood Assay, a new method for endotoxin level measurement, has been improved and evaluated to allow the quantitative analysis of hemodialysis liquids and biofilms. It has been shown to be sensitive and reproducible and those first results highlighted the hypothesis that this test allows detection of most "endotoxin-like" components. Presently, the LAL assay is the only standard method for the control of the microbiological quality of hemodialysis systems but our data suggests that the WBA has a promising future in diagnosing endotoxin contamination in hemodialysis systems.

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