

Methods for Biofilm Analysis on Silicone Tubing of Dialysis Machines

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Abstract: We describe an analytical protocol to study biofilms that develop inside silicone tubing of dialysis machines. This protocol has been set up with the help of a dynamic testing device reproducing dialysis conditions. The methodology includes direct microscopic observation, biofilm removal with an original mechanical biofilm scraper, quantitative analysis with culturable and total bacteria counting, and endotoxin level measurement using the LAL chromogenic kinetic assay. The analytical protocol has been assessed on 13 different clinical tubing samples. Most samples were contaminated by adherent cells and the thickest biofilms were found at the connection between the

dialysis water distribution loop and the dialysis machine. The less contaminated samples had been removed from dialysis machines that were decontaminated with citric acid and autoclaving, showing the importance of the decontamination procedure for the prevention of biofilm development. This article shows that easy, rapid, reproducible, and economical methods are applicable for a routine analysis of biofilms that develop on dialysis systems and should be included in the regular control of the microbiological quality of dialysis liquids. **Key Words:** Biofilm—Hemodialysis—Analysis—Endotoxin—Silicone tubing.

Bacteria stick firmly to almost any material in contact with an aqueous environment to form biofilms. These sessile communities and their inherent resistance to antimicrobial agents lead to many environmental problems and bacterial diseases (1,2). Biofilm development may even occur in distribution systems where water contains very low levels of organic compounds. In fact, Man et al. demonstrated that such biofilms can develop on the inner surface of tubing from dialysis fluid pathways (3). This represents a significant health risk for patients despite periodic microbiological analysis of water and dialysate. This is because of biofilm sloughing, which can cause significant, but unpredictable, release of bacteria and bacterial endotoxins. Nowadays, most hemodialysis centers check the contamination of dialysis water and quite a few also control the dialysate, but biofilm development inside dialysis

machines is not taken into account. Moreover, tubing replacement is not always performed. When the replacement is performed, the frequency and extent of such a replacement has not been clearly defined or documented.

The objective of the work discussed here is to develop a procedure for the assessment of microbial growth on silicone tubing from hemodialysis apparatus. The methods are designed to be easy and rapid so as to be clinically acceptable, allowing both immediate qualitative results as well as total and viable bacterial counts.

MATERIALS AND METHODS

Dynamic testing device

Two bacterial strains were isolated from a contaminated dialysate from a dialysis clinic. The bacteria were identified as *Pseudomonas paucimobilis* and *Pseudomonas picketti*, using an automatic identification system, Mini-API 99813, with identification galleries ID 32 E and ID 32 GN (Biomérieux, France). Three other strains were purchased from the Pasteur Institute collection: *Pseudomonas*

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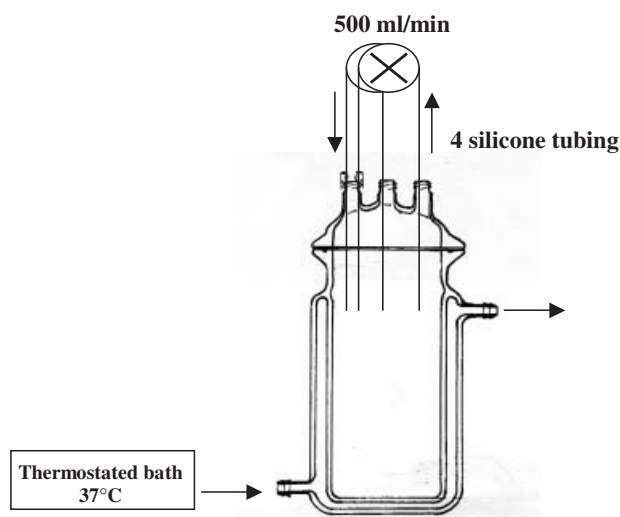


FIG. 1. Dynamic testing device.

aeruginosa A22, *Aeromonas hydrophila* 103697, and *Bacillus subtilis* 5265. Each strain was grown separately on LB broth (Difco, France) until in stationary phase. The cultures were washed twice with sterile and pyrogen-free water to remove organic materials.

Dialysate solution was prepared from sterile concentrates (Clearflex, Bieffe Medital, Sabinanigo, Spain) diluted with sterile and pyrogen-free water. Two hundred milliliters of this dialysate was inoculated with about 10^6 CFU/ml of each strain. This contaminated dialysate was poured into the main part of a reactor that fed four samples of silicone tubing, each 60 cm in length with an inner diameter of 5 mm (Masterflex 7015, Bioblock Scientific, France; Fig. 1). The flow rate (500 ml/min) was controlled by a four-headed peristaltic pump (Masterflex L/S, Bioblock Scientific). The external compartment of the reactor was filled with distilled water kept at 37°C by a thermostated bath (Polystat 22, 86602, Bioblock Scientific).

Biomass removal

For the quantitative analyses, it was necessary to remove the biomass from the inside of the tubing. A new mechanical removal method was constructed for entire biomass removal from the tubing in a rapid and reproducible way. It consists of a stainless steel spatula (5 or 8 mm $\times$ 5 cm) driven by an electric screwdriver (Versapack, Black & Decker, France). The outer surface of a 5 cm tubing sample was disinfected with a 70% alcohol solution and the inner surface was rinsed twice with sterile and pyrogen-free water. The tubing was placed into a punched cap of a sterile tube (TPP, Fisher Bioblock, France). The

spatula turned regularly inside the tubing and detached the biofilm. The biofilm was then collected in the sterile tube with the help of a slow 10 ml water rinse. Bacterial aggregates were then dissociated through the needle of a syringe. This mechanical scraper allowed the entire biofilm removal in 1 min on a 5 cm tubing sample. This "biofilm scraper" is shown in Fig. 2.

A sonication bath (frequency: 48 kHz, power 120 W; Branson 5200, Branson, U.S.A.) was used for comparison experiments between ultrasonic treatment and scraping method. Five tubing samples were placed in 30 ml sterile and pyrogen-free water into an ultrasonic bath for 5, 10, 15, 20, 25, and 30 min, respectively. Then, each tubing sample was scraped so that each sample was its own control. Culturable bacteria were enumerated from the aqueous solutions resulting from both treatments.

Staining methods

Direct and indirect staining methods using light (Nikon Alphaphot YS) or epifluorescence (Olympus BX 60) microscopy were performed for qualitative

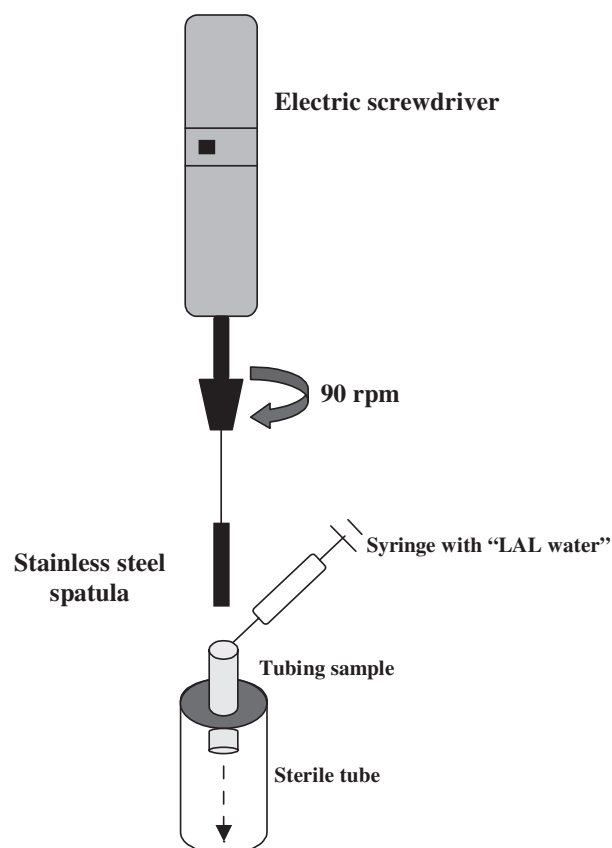


FIG. 2. Mechanical biofilm scraper.

TABLE 1. Characteristics of the tubing samples removed from dialysis systems

#	Center	Removing site	Internal diameter (mm)	Total running time (months)	Decontamination method
1	b	Control (new, unused)	4	0	No decontamination
2	b	Control (new, unused)	4	0	No decontamination
3	c	Connection loop-dialysis machine	9	12	No decontamination
4	c	Connection loop-dialysis machine	8	12	No decontamination
5	c	Connection loop-dialysis machine	6	12	No decontamination
6	a	Inside dialysis machine	4	12	C
7	a	Inside dialysis machine	5	12	C
8	a	Inside dialysis machine	4	12	C
9	a	Exit dialysis machine	6	12	A
10	a	Exit dialysis machine	6	12	A
11	a	Exit dialysis machine	6	12	C
12	a	Exit dialysis machine	6	12	A
13	a	Exit dialysis machine	6	12	C

A, citric acid 3%, 37°C, 5 minutes + autoclaving 121°C, 30 min; C, chemical decontamination (Dialox 0.3%, Actril 0.3%, or bleach 0.25%).

and quantitative analysis of the biofilm. Aqueous solutions of crystal violet (CV) 0.25% (Sigma, France), and DAPI 5 µg/ml (Sigma) were prepared.

The tubing was stained with crystal violet to check biomass removal after scraping and for a direct observation of the biofilm on the surface of the tubing to monitor biofilm development. The samples were immersed in a 0.25% CV aqueous solution for 5 min, rinsed twice with distilled water, cut lengthwise, air dried, fixed with adhesive tape on a glass slide, mounted with immersion oil, and observed by light microscopy. This method allowed a rapid qualitative analysis of biofilm growth with direct visualization of the entire components of the biofilm.

DAPI staining was performed on the suspension formed from the tubing scraping for total bacteria enumeration. This solution was run through a black polycarbonate membrane (Isopore, pore size 0.45 µm, 13 mm ID, Millipore, France). The membrane was rinsed and placed on the epifluorescence microscope and a total bacterial count was obtained. This method was performed according to the technique described by Schaule (4).

Culturable bacteria counting

Determination of culturable bacteria in samples from detached biofilms was assayed by plate counts. Samples were serially diluted and spread using R₂A agar medium (Difco). The CFU were counted after a 7 day incubation time at 20°C.

Quantification of lipopolysaccharides

Lipopolysaccharides (LPS) were quantified by using the LAL chromogenic kinetic assay (Endochrome-K, Charles River Endosafe, France).

The standard procedure described in the European Pharmacopoeia text #2614 addendum 2001 was used on 100 µl bacterial suspension samples (5). The kinetic analyses were performed with a microtube reader (ATI 320, Charles River Endosafe) with an onset OD of 0.100. The LPS from *E. coli* 055B5 was used to calibrate the method.

Application to silicone tubing removed from dialysis machines

Once developed, the whole analytical protocol was applied for the analysis of 13 different clinical samples of silicone tubing removed from dialysis systems in three French dialysis centers. The different samples are described in Table 1. As an example, Fig. 3 illustrates the internal circuit of a dialysis machine where the different samples were removed.

RESULTS

Evaluation of biofilm removal technique

The mechanical scraping technique was tested for its ability to remove biofilm. The CV staining method was applied subsequent to scraping to check the complete removal of the biomass. The method was tested on both a thin 1-month-old biofilm and a thick 2-year-old biofilm. In both cases, light microscopic observation showed no more residues (Fig. 4).

Comparison experiments between the mechanical scraping and the ultrasonic bath showed that after a 5 min sonication time, the bacterial count was only 60% of that obtained by the scraping technique. Even after a 30 min sonication time, bacterial counts were only 90% of those from the mechanical technique.

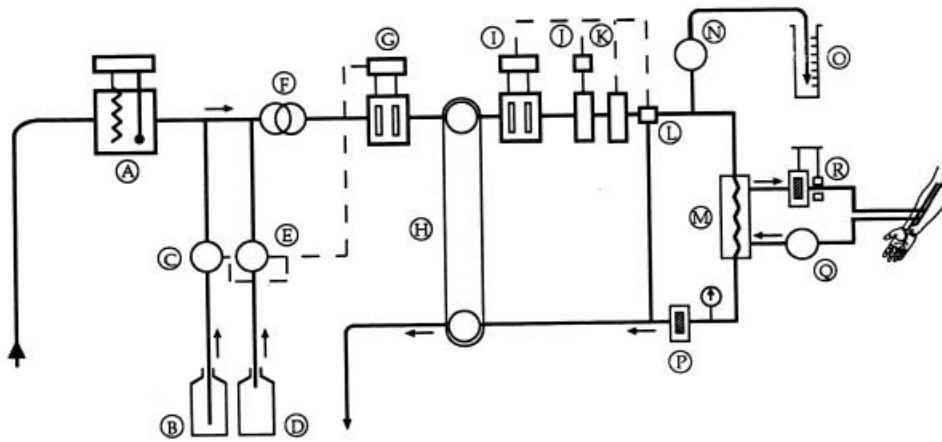


FIG. 3. Internal circuit of a dialysis machine. A, heater; B, acid concentrate; C, pump for acid concentrate; D, bicarbonate concentrate; E, pump for bicarbonate concentrate; F, gas pump; G, conductivity meter; H, volumetric pump; I, conductivity check; J, temperature check; K, dialysate flow meter; L, shunt; M, dialysis membrane; N, ultrafiltration pump; O, ultrafiltrate sampler; P, blood leaks detector; Q, blood pump; R, air detector.

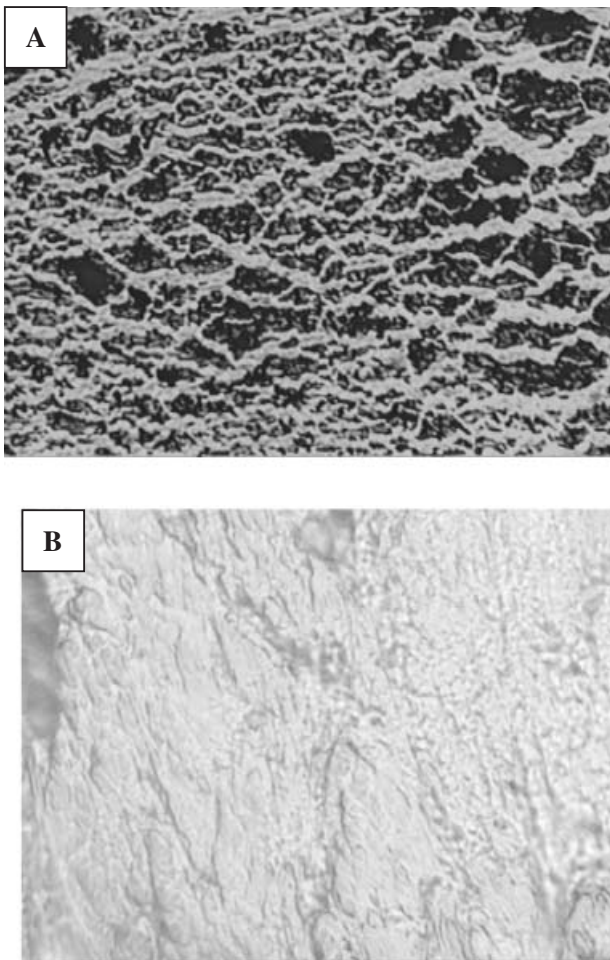


FIG. 4. Efficacy of biofilm removal with the mechanical scraper. (A) Mature biofilm before scraping. (B) Clean inner surface after scraping.

Assessment of biomass formed on the silicone tubing from the dynamic testing device

Biofilm development in the four tubes was qualitatively followed by CV staining for 50 days. Direct observation of the stained tubing samples showed an increase in the coloration intensity within this observation period. This method allowed the observation of the different biofilm formation steps. After 7 days, the biofilm consisted of isolated sessile bacterial cells. Dispersed microcolonies were observed after 2 weeks. The 1-month-old biofilm included single cells and microcolonies embedded in an exopolysaccharide matrix. Pictures of those different formation steps are shown in Fig. 5.

Biofilm development was also followed quantitatively by taking tubing samples on a regular basis and using the scraping, CFU, total bacteria, and endotoxin level measurements described previously.

The results from these analyses indicate a biofilm containing more than 10^5 bacteria/cm² within 1 month, as shown in Table 2.

TABLE 2. Quantitative assessment of biofilm growth for 50 days, on the silicone tubing removed from the dynamic testing device including culturable and total bacteria counting and endotoxin level measurement

Day	CFU ×10 ⁴ /cm ²	Total bacteria ×10 ⁴ /cm ²	Endotoxin (EU/cm ²)
0	0.00	0.00	0.00
3	0.01	0.20	0.08
7	0.50	1.00	0.70
10	1.30	6.20	1.00
14	1.30	8.00	2.50
17	2.00	12.00	3.10
21	2.50	20.00	5.00
30	38.00	60.00	10.00
38	39.00	65.00	11.00
50	41.00	67.00	10.50

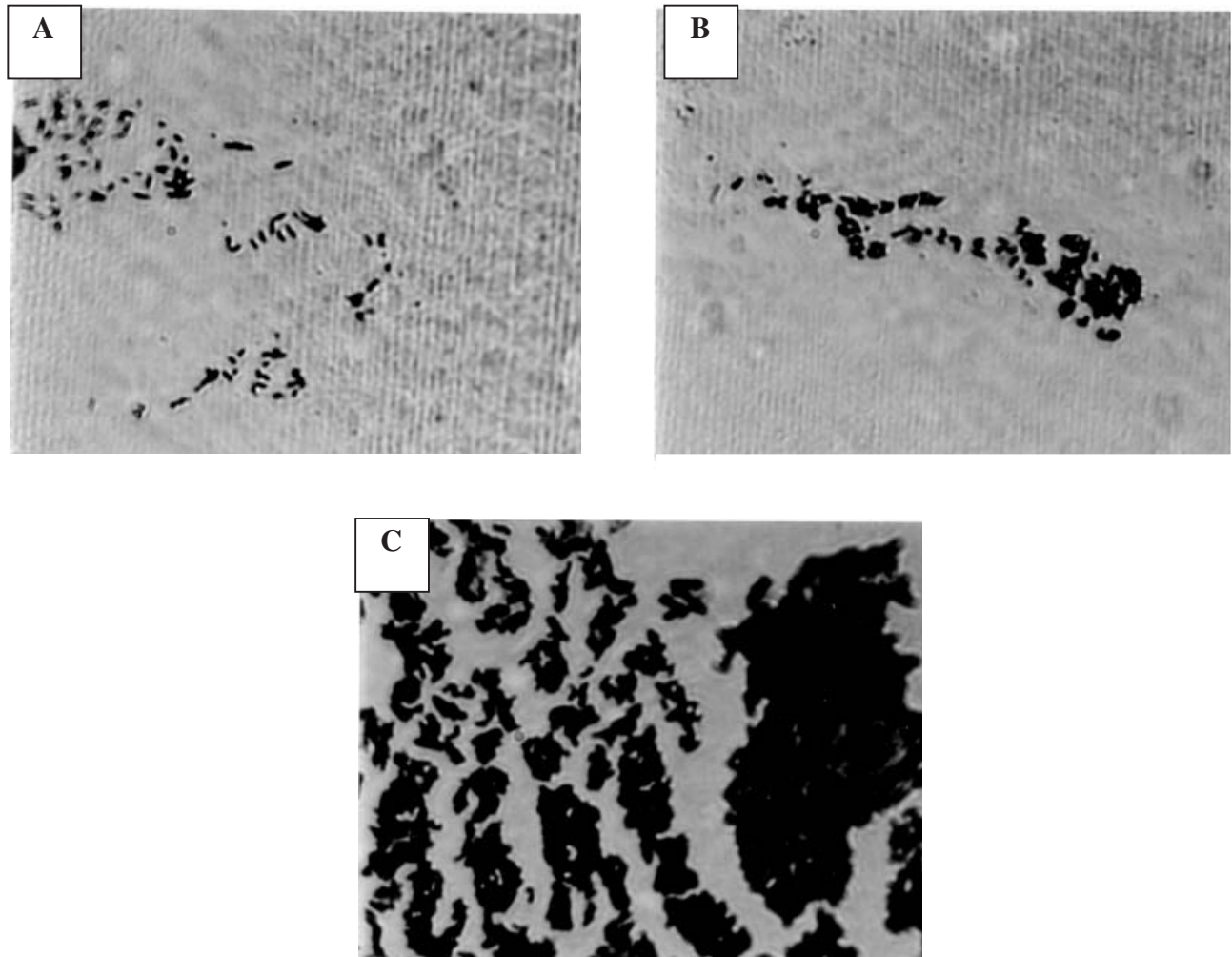


FIG. 5. Different biofilm formation steps. (A) First adherent cells (day 7). (B) Dispersed microcolonies (day 14). (C) Mature biofilm (day 30).

Application to clinical samples

Results for the 13 clinical samples are given in Table 3. No tubing sample, except for the two controls, was completely clean. Crystals were almost always present on the inner surface of the tubing with isolated adherent bacteria or microcolonies. The main bacterial strains isolated were *Pseudomonas* sp., *Bacillus* sp., *Xanthomonas maltophilia*, and *Aeromonas salmonicida*, as determined by Mini-API analyses.

These results indicate that the decontamination method is the determining factor in the amount of biofilm. Tubing that underwent a citric acid pre-treatment followed by autoclaving had less contamination. Conversely, tubing removed from the connection between the water distribution loop and the dialysis machine was contaminated with a thick, extensive, mature biofilm.

DISCUSSION AND CONCLUSION

This analytical procedure, based on an original scraping method associated with microscopic analysis, was easy to perform, rapid, economical, and efficient to study biomass formation on hemodialysis silicone tubing samples. The complete analysis of ten tubing samples was performed within 1 day (excluding the growth time for the culture sample).

Complete detachment and disaggregation of the biofilm was achieved by using the mechanical scraper in combination with repeated syringing. These observations are in good agreement with Costerton's investigations, which point out the importance of a mechanical removal of the biofilm prior to microbiological analysis (6). Our results suggest that this method was reproducible and efficient in removing biofilms from their supports. Moreover, comparison

TABLE 3. *Qualitative and quantitative analysis of the 13 clinical samples*

#	Microscopic observation	Total bacteria/cm ²	Culturable bacteria CFU/cm ²	Endotoxins EU/cm ²
1	Clean	180	<1	<0.005
2	Clean	115	<1	<0.005
3	Mature biofilm	5.3×10^6	9.7×10^5	354
4	Mature biofilm	1.2×10^5	1.6×10^3	115
5	Mature biofilm	1.3×10^5	358	89
6	Microcolonies + crystals	4,550	11	0.3
7	Mature biofilm	3.0×10^5	133	3.7
8	Microcolonies	2.0×10^4	81	1.9
9	Crystals + isolated bacteria	2,130	4	0.05
10	Crystals + isolated bacteria	1,335	8	0.01
11	Microcolonies + isolated bacteria	6,400	38	0.2
12	Crystals + isolated bacteria	530	7	0.1
13	Microcolonies + crystals	4,000	13	0.2

experiments between this technique and ultrasonic treatment showed that the scraper gave the best results. Ultrasonic treatment depends on ultrasonic apparatus characteristics and is often used to kill bacteria in order to produce endotoxins. Therefore, it could interfere with an accurate assessment of the biomass.

Staining methods for microscopic observations give rapid results, allowing the following of the evolution of the biofilm in the process of time and the enumeration of total bacteria after biofilm detachment. Costerton and Grimont also recommend direct microscopic observation to study the microbial contamination of water samples and biofilms (6,7). These methods can easily replace scanning electron microscopy, which is expensive, time consuming, and only allows observation of very small surfaces. Nevertheless, these microscopic methods cannot give any information concerning the pathogenicity of the bacteria. So, plate counts and biochemical identification of strains must be carried out.

The principal health risk related to hemodialysis fluid contamination is represented by bacterial pyrogens, mainly endotoxins that are known to cross dialysis membranes (8). Endotoxin level measurement is an essential parameter that must be taken into account for the analysis of biofilms from dialysis machines.

This protocol was developed with clinical analyses in mind and should be recommended for routine evaluation of dialysis systems.

The results for 13 clinical tubing samples are in agreement with Man's investigation, which described the presence of biofilms inside silicone tubing with a large amount of calcium and magnesium carbonate crystals (3). These crystals are coming from the dialysate and adhere firmly to the surface of the tubing, likely increasing the adherence of the bacte-

ria and accelerating biofilm development. Our results highlight the importance of a descaling step in the decontamination procedure. Moreover, one decontamination procedure based on the association of citric acid and autoclaving seems to be efficient for the prevention of biofilm development.

Additional investigations are under way to improve on these methods. For instance, the LAL assay, which is the only test available for routine analysis, has some disadvantages. This test only detects entire LPS or lipid A, but according to many authors, this is only a fraction of the compounds released from bacteria that have pyrogen properties (9–11). Tests based on inflammatory factors released by mononuclear cells are more efficient for a global measurement of pyrogen contamination (10,12). A new test using human whole blood is currently in process in our laboratory and gives promising results.

A method based on the measurement of optical densities on microplates for direct biofilm quantification is also in development.

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