Effects of biofilm formation on haemodialysis monitor disinfection

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Abstract

Background. Biofilms are composed of communities of micro-organisms adhering to essentially any surface. We evaluated whether biofilm formation in the hydraulic circuit of a purposely contaminated haemodialysis monitor would modify the efficacy of different disinfection modalities against bacteria and endotoxin concentrations.

Methods. A water-borne Pseudomonas aeruginosa (109) suspension was recirculated for 1 h and was left standing for 72 h (stationary phase) in the hydraulic circuit of the monitor. The monitor was then washed and disinfected by different physical (heat, 85°C) or chemical (hypochlorite or peracetic acid) disinfection modalities (protocol A). In protocol B, the bacterial suspension was also recirculated for 1 h, but the monitor was then immediately washed and disinfected by different chemical disinfection modalities (hypochlorite or peracetic acid).

Results. Biofilm formation was revealed by scanning and confocal laser electron microscopy after the stationary phase (protocol A), but was absent when the monitor was immediately washed and disinfected (protocol B). In the presence of biofilm (protocol A), heat in association with citric acid was the most effective modality for reducing both colony forming units and endotoxin concentrations, whereas heat by itself was the least effective method of disinfection. Dwelling (60 h) with diluted peracetic acid completely prevented the formation of biofilm. In the absence of biofilm (protocol B), chemical disinfection proved to be effective against both colony forming units and endotoxin concentrations.

Conclusions. We found that biofilm formation may markedly reduce the efficacy of presently available disinfection modalities. Therefore, different disinfection modalities and the combined action of descaling (by citric acid) and disinfection (physical/chemical agents) should be used periodically in haemodialysis monitors. In addition, dwelling with diluted peracetic acid should be adopted whenever monitors are not in use.

Keywords: biofilm; contamination; cytokine; disinfection; microinflammation; monitor

Introduction

Biofilms are composed of communities of micro-organisms that adhere to essentially any surface. Biofilms have been described as highly structured habitats with extracellular polymer matrix structures that encase bacteria. Several mechanisms are involved in the formation of biofilms [1–4]. Of relevance for haemodialysis, biofilm formation represents the starting point for biofouling, resistance to antibiotic chemotherapy or disinfection and bacterial regrowth [5].

In the haemodialysis system, favourable sites for biofilm formation include the water treatment system, the distribution pipelines and the hydraulic circuit of the dialysis monitor, because of contamination by water-borne bacteria, the presence of organic nutrients and high pH due to bicarbonate-buffered solutions. In addition, physical factors, such as dead ends, low fluxes and no flow periods, may favour biofilm formation [6–9].

Although the nephrology community has been provided with information relating to effects of disinfectants on bacterial suspensions, there is little data on bacterial and endotoxin reappearance. For the disinfection of haemodialysis monitors, several chemical, physical and combined chemical–physical treatments have been introduced in routine practice. Chemical agents include disinfectants, such as chlorine-based products, aldehyde derivatives and peracetic acid. Physical agents include the use of ultraviolet irradiation, heat at varying maximal temperatures and steam.
Furthermore, descaling is performed with various acids (acetic, lactic or citric) either alone or in combination with disinfectants.

In haemodialysis, the efficacy of disinfection in the presence of biofilms has never been precisely evaluated. Persistent bacteria encased in biofilms may cause recurrent passage of cytokine-inducing substances into the bloodstream, leading to chronic inflammation [10–12].

The present studies were designed to evaluate whether biofilms, formed in the hydraulic circuit of a purposely contaminated haemodialysis monitor, would affect the efficacy of different physical and chemical disinfection modalities on colony forming units (CFU) and endotoxin concentrations.

Subjects and methods

Preparation of bacterial suspensions

Fresh bacterial suspensions were obtained from water-borne Pseudomonas aeruginosa in the log phase of growth and were cultured on cetrimide agar medium at 37°C for 24 h. The bacterial count was set to nearly 10⁹ CFU/ml by using optical density.

Experimental set-up

Haemodialysis monitor. A commercially available haemodialysis monitor was used for these studies. The monitor had a single-pass hydraulic circuit (total volume: 1300 ml) with a flow rate of 40 cm/s at 500 ml/min for an average section of 0.2 cm² (Figure 1). An additional recirculation circuit was added to the conventional circuit to recirculate the bacterial suspension and to promote the formation of the biofilm (see below for protocols A and B).

We aspirated 20 ml from each of the different sampling sites (Figure 1) at 0, 24, 48 and 72 h after disinfection. We then filtered 18 ml (0.45 μm) and incubated this aerobically in cetrimide agar medium. Bacteria were counted after 24 h at 36°C and endotoxin concentrations.

Table 1. List of disinfectants categorized according to the active compound

<table>
<thead>
<tr>
<th>Disinfectant</th>
<th>Manufacturer</th>
<th>Concentration (mg/l)</th>
<th>Contact time (min)</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active chlorine</td>
<td>Hypochlorite, C. Erba (Milan)</td>
<td>3333</td>
<td>6</td>
<td>37</td>
</tr>
<tr>
<td>Peracetic acid</td>
<td>Henkel EcoLab (Agrate B, Milan)</td>
<td>1416</td>
<td>6</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>Air Liquide Sanità (Milan)</td>
<td>2300</td>
<td>3600</td>
<td>RT*</td>
</tr>
<tr>
<td>Heat</td>
<td>–</td>
<td>–</td>
<td>15</td>
<td>85</td>
</tr>
<tr>
<td>Heat +</td>
<td>–</td>
<td>–</td>
<td>15</td>
<td>85</td>
</tr>
<tr>
<td>Citric acid</td>
<td>C. Erba (Milan)</td>
<td>20 000</td>
<td>6</td>
<td>85</td>
</tr>
</tbody>
</table>

*RT, room temperature.

To rule out the presence of disinfectants, all samples were tested for disinfectant residues (for hypochlorite: the Chlorine and pH Test, Merck, Darmstadt, Germany, sensitivity: 0.1 p.p.m.; for peracetic acid: the Peracetic acid Test, Merck, 5 p.p.m.; for peroxides: the Peroxides Test, Merck, 0.5 p.p.m.). Only samples that were negative for residues were used for further testing. Each disinfection protocol was preceded by washing with deionized water for 5 min at 500 ml/min. For heat disinfection, tank temperature was raised to 95°C to obtain an average temperature of 85°C in the circuit and the heated water was circulated single-pass at 250 ml/min (Table 1).

Protocol A

The monitor was uniformly contaminated by recirculating 500 ml of the bacterial suspension (P. aeruginosa, 10⁹ CFU/ml) in 1 l isotonic saline for 1 h at 37°C (Figure 2). The monitor was then switched off, leaving the bacterial suspension inside the circuit for 72 h (stationary phase). The monitor was then washed with deionized water for 5 min at 500 ml/min. In selected experiments, the contaminated monitor was disinfected with heat + citric acid and dwelling with 0.37% peracetic acid. The monitor was turned off and after 60 h of non-use, samples were taken at 0, 24, 48 and 72 h.

The monitor automatically diluted the chemical disinfectants (hypochlorite 10% or peracetic acid 4%) and citric acid 12% as the descaling agent at 1 : 30 and 1 : 6 dilutions, respectively.

Protocol B

This procedure was the same as for protocol A, except that the stationary phase was omitted and the monitor was immediately washed and disinfected with hypochlorite and peracetic acid (Figure 2).

Scanning electron microscopy (SEM) studies

Segments of the hydraulic circuit were fixed in glutaraldehyde (2%) for 20 min, washed in phosphate-buffered saline and dehydrated through 30, 50, 75, 90 and 100% ethanol series, allowing 30 min in the lower concentrations and progressing to 60 min in the higher concentrations. They were then dried at the critical point of carbon dioxide and coated with gold–palladium. Prepared samples were scanned with a Philips XL-40 scanning electron microscope.
Confocal laser scanning microscopy (CLSM) studies
Silicon segments (1 cm²), obtained from the hydraulic circuit 72 h after the stationary phase (protocol A), were incubated with nuclear DNA staining fluorochromes, SYTO 9 and propidium iodide for 10 min at room temperature (LIVE/DEAD BacLight; Molecular Probes, Eugene, OR, USA). Using a LEICA TCS-4D, membrane-permeable SYTO 9 labelled live bacteria in green, while membrane-impermeable propidium iodide stained dead bacteria in red.

Statistical analysis
The data from three experiments in protocol A and four experiments in protocol B were organized in a relational database and analysed by a statistical package (SPSS; Microsoft Corp, Redmond, VA, USA). Student’s t-tests or variance analysis were performed, when appropriate, to analyse pre- and post-disinfection values or to compare disinfectants. During analysis, CFU values below detection limits were assigned a value of 0.025 CFU/ml. Differences between groups were considered significant at $P < 0.05$.

Results
In protocol A, SEM analysis showed (Figure 3A) an early (24 h) bacterial adhesion to silicone tubings. After 72 h of the stationary phase, average CFU values were reduced, albeit not significantly ($P = 0.2646$). At
the same time point, SEM analysis revealed the presence of bacteria appearing as a sludge adhering to the silicon (Figure 3B), as well as biofilm formation that surrounded and glued together bacterial formations (Figure 3C).

CLSM studies revealed a widespread deposition of live bacteria (green fluorescence) combined with a low background of dead bacteria (red fluorescence) (Figure 4).

**Protocol A: effect of disinfection on CFU and endotoxin formation**

Treatment with the different disinfection modalities led to a highly significant reduction in CFU at all time intervals and sampling points ($P = 0.0001$) (Table 2).
Table 2. Bacteria (CFU/ml) and endotoxin (EU/ml) in the haemodialysis monitor evaluated according to protocol A with different disinfection modalities. Values are the means ± 1 SD of three experiments for each disinfection modality.

<table>
<thead>
<tr>
<th>Sampling timing</th>
<th>Hypochlorite CFU/ml</th>
<th>Hypochlorite EU/ml</th>
<th>Peracetic acid CFU/ml</th>
<th>Peracetic acid EU/ml</th>
<th>Heat CFU/ml</th>
<th>Heat EU/ml</th>
<th>Heat + citric acid CFU/ml</th>
<th>Heat + citric acid EU/ml</th>
<th>Heat + citric acid + dwelling CFU/ml</th>
<th>Heat + citric acid + dwelling EU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-disinfection</td>
<td>2.25 × 10⁸ ± 5.00 × 10⁷</td>
<td>1615 ± 10⁴</td>
<td>1.65 × 10⁸ ± 1.08 × 10⁷</td>
<td>1233.25 ± 203.72</td>
<td>7.25 × 10⁶ ± 1.71 × 10⁶</td>
<td>1312.5 ± 33 ± 57.3</td>
<td>2.50 × 10⁸ ± 1.00 × 10⁶</td>
<td>1620 ± 109.5 ± 129.87</td>
<td>2.75 × 10⁸ ± 5.00 × 10⁷</td>
<td>1490 ± 96.7 ± 0.025</td>
</tr>
<tr>
<td>0 h post-disinfection</td>
<td>177.5 ± 5</td>
<td>97.5 ± 4.5</td>
<td>112.5 ± 1.7</td>
<td>6.3 ± 0.6</td>
<td>0.025</td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h post-disinfection</td>
<td>1575 ± 24.2</td>
<td>255 ± 88.6</td>
<td>1550 ± 21.6</td>
<td>14.3 ± 8.9</td>
<td>0.025</td>
<td>1.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>48 h post-disinfection</td>
<td>5675 ± 90</td>
<td>900 ± 46.6</td>
<td>5250 ± 1215.2</td>
<td>6.9 ± 11.7</td>
<td>0.025</td>
<td>1.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>72 h post-disinfection</td>
<td>6000 ± 109.5</td>
<td>1462.5 ± 292.2</td>
<td>9250 ± 167.3</td>
<td>72 ± 47.8</td>
<td>0.025</td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

There were no differences between the different sampling points at any of the time intervals (P = 0.2832). The association of heat with citric acid resulted in the highest reduction in CFU (at 0 h: 6 ± 5 CFU/ml; P = 0.0008 vs all other disinfection modalities at each sampling time). In contrast, heat by itself showed a much lower disinfection effect compared with hypochlorite and peracetic acid at all time points (P = 0.0001). Hypochlorite and peracetic acid had similar effects on CFU (P = 0.106) (Table 2).

Bacterial regrowth after disinfection at 72 h (vs 0 h values) was 30-fold higher with hypochlorite and 80-fold higher with heat (Table 2). With peracetic acid, bacterial regrowth increased, but to a much lesser extent (~15-fold over post-disinfection values; P = 0.0234). The association of heat with citric acid induced the lowest regrowth of bacteria (~10-fold; P = 0.0312) (Table 2). Bacterial regrowth was completely abrogated after heat with citric acid followed by dwelling (60 h) with diluted peracetic acid (Table 2).

Post-disinfection reappearance of endotoxin occurred, but with a kinetics that was different from that described for CFU. Maximal reappearance occurred after disinfection with heat (100-fold higher than post-disinfection values). All of the other disinfection modalities produced endotoxin reappearance at varying magnitudes vs post-disinfection values. For example, heat with citric acid, peracetic acid and hypochlorite resulted in 90-, 50- and 20-fold increases, respectively. However, mean values of endotoxin concentrations at 72 h revealed that heat in association with citric acid resulted in the lowest endotoxin levels in all samples, which contrasted with peracetic acid and heat disinfection (P = 0.008 vs peracetic acid; P = 0.024 vs heat), but not with hypochlorite (P = 0.191). Importantly, endotoxin reappearance was completely abrogated after 60 h of dwelling with diluted peracetic acid (Table 2).

Protocol B: effect of disinfection on CFU and endotoxin

In experiments without biofilm formation, there were no significant differences between the different sampling points at any given time interval (P = 0.241) (Table 3). Hypochlorite and peracetic acid significantly (P = 0.0001) reduced both CFU and endotoxin levels (Table 3).

Comparative analysis of LRV in protocols A and B

Figure 5 shows mean LRV for CFU (A) and endotoxin (B) concentrations in protocols A and B with the different disinfection modalities. In protocol A, hypochlorite and peracetic acid resulted in reduced LRV for CFU (<6 from 24 h on), whereas heat was remarkably ineffective even as early as 0 h (LRV = 4.96). Only heat in association with citric acid produced a persistently high LRV for CFU (>6). In protocol B, hypochlorite and peracetic acid generated high LRV for CFU (LRV > 6) (Figure 5A). The association of heat with citric acid resulted in significantly larger LRV for endotoxin than with peracetic acid after disinfection (P = 0.0008) (Figure 5B) and this difference persisted to the end of the study. In addition, hypochlorite and peracetic acid produced significantly different LRV starting at 24 h and thereafter (data not shown). After all disinfection modalities, a significant decrease in LRV for endotoxin was obtained at 72 h compared with post-disinfection values (P = 0.008). Heat associated with citric acid had a higher LRV than heat alone (at 72 h: P = 0.0008) (Figure 5B).

Discussion

In the present studies, we formed biofilms in a haemodialysis monitor and evaluated the efficacy of
conventional sterilization techniques. These studies showed that biofilm formation significantly reduced the efficacy (expressed as LRV for both CFU and endotoxin; Figure 5) of both physical and chemical disinfection agents. As shown in Table 2, heat alone in the presence of biofilm produced the lowest LRV for CFU (3.79) compared with values from well-known chemical disinfectants, such as hypochlorite and peracetic acid (5.22 and 5.57, respectively). However, for bacterial growth in the presence of biofilm, only heat in association with citric acid caused reductions in growth rates (LRV = 7.06). All disinfectants were significantly less effective for endotoxin reappearance. Importantly, there was neither bacterial regrowth nor endotoxin reappearance after heat in association with citric acid followed by 60 h dwelling with diluted peracetic acid.

To our knowledge, this is the first study to examine biofilm formation and to test the effects of different disinfection modalities under carefully controlled conditions. We used a single-pass circuit (without a recirculation loop), allowing a high flow rate.

The present studies had certain limitations. For example, the accelerated biofilm formation may not be representative of biofilms that develop under ‘real’ conditions. This is because the slow development of ‘natural’ biofilm may be associated with the formation of matrix and altered gene expression, rendering ‘natural’ biofilm different from our surrogate. In clinical practice, the levels of contamination are much lower than in the present study and formation of bacterial biofilm may require several days or even weeks. To date, there are no methods for directly assessing the presence of biofilm, except when its presence is presumed on the basis of persistent endotoxin contamination.

In the presence of biofilm (protocol A), all the disinfection modalities (hypochlorite, peracetic acid and heat) were incapable of completely eliminating CFU and endotoxin (Table 2). With each of these modalities, bacterial regrowth and endotoxin reappearance started immediately after disinfection, they increased to a maximum at 24 h and reached a plateau at 48 h (data not shown). The maximal bacterial growth was probably

**Table 3.** Bacteria (CFU/ml) and endotoxin (EU/ml) in the haemodialysis monitor evaluated according to protocol B with different disinfection modalities. Values are means ± 1 SD of four experiments for each disinfection modality.

<table>
<thead>
<tr>
<th>Sampling timing</th>
<th>Hypochlorite CFU/ml</th>
<th>EU/ml</th>
<th>Peracetic acid CFU/ml</th>
<th>EU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-disinfection</td>
<td>9.750 × 10^6 ± 5.14 × 10^6</td>
<td>1213.5 ± 210.7</td>
<td>9.57 × 10^7 ± 4.83 × 10^7</td>
<td>1380 ± 38.5</td>
</tr>
<tr>
<td>0 h post-disinfection</td>
<td>4.0 ± 4.5</td>
<td>1.1 ± 0.4</td>
<td>26 ± 8.6</td>
<td>2.1 ± 0.6</td>
</tr>
<tr>
<td>24 h post-disinfection</td>
<td>3.8 ± 4.8</td>
<td>6.5 ± 1.8</td>
<td>3.0 ± 2.9</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>48 h post-disinfection</td>
<td>2.8 ± 4.9</td>
<td>5.1 ± 3.2</td>
<td>3.8 ± 2.2</td>
<td>3.0 ± 1.6</td>
</tr>
<tr>
<td>72 h post-disinfection</td>
<td>5.3 ± 5.5</td>
<td>3.5 ± 1.0</td>
<td>3.8 ± 1.9</td>
<td>1.9 ± 1.4</td>
</tr>
</tbody>
</table>

**Fig. 5.** Mean (+1 SD) LRV values for CFU (A) and endotoxin (B) concentrations in protocols A (open bars) and B (closed bars) with different disinfectants. (A) *P = 0.0017, †P = 0.0179, ‡P = 0.0410; (B) *P = 0.0286, n.s., not significant.
limited by the amount of nutrients present in the dialysate. However, as expected, endotoxin concentrations continued to increase even after 24 h. Under these conditions, bacterial regrowth and endotoxin reappearance were completely abrogated after heat in association with citric acid followed by 60 h dwelling with diluted peracetic acid. In the absence of biofilm (protocol B), both hypochlorite and peracetic acid displayed full bactericidal activity, as indicated by very high LRV for CFU (in both cases >6). Nevertheless, LRV for endotoxin was markedly lower (≤3).

Our findings might provide indications for elimination of biofilms once they are formed in haemodialysis monitors. For example, we would recommend the periodic application of descaling combined with disinfection (both physical and chemical agents). The action of acid descaling might allow disinfectants to penetrate more deeply into the highly structured habitat of the biofilm, since disinfectants alone might act only at the surface of the biofilm. The practice of disinfectant dwelling has been proposed for periods of non-use in haemodialysis monitors [12]. In agreement, our present data indicate that this may provide a reliable measure to prevent the formation of biofilm. Furthermore, our data would advocate the association of different disinfection modalities, particularly the adoption of chemical (hypochlorite or peracetic acid) or physico-chemical (heat + citric acid) disinfections. However, physical disinfection modalities, such as the heat, previously proposed for environmental benefits, should be used with great caution since this method was associated with the highest regrowth and endotoxin concentrations. In fact, our data are in agreement with others that also showed unreliable disinfecting effects of heat [13]. In our opinion, the use of heat should be confined to disinfections applied between dialysis sessions. However, this does not apply to disinfection modalities that use much higher temperatures (120°C).

In conclusion, the formation of a ‘surrogate’ biofilm had a remarkable adverse influence on the efficacy of different disinfection modalities. These studies emphasize concerns about the prevention and elimination of biofilms in haemodialysis monitors. Routine methodologies to detect biofilm in monitors should be made available to determine true prevalence rates and to aid in successful elimination of biofilms and their proinflammatory potential [14,15].

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Conflict of interest statement. L. Sereni, M. Morselli, M. Bellesia and C. Tetta were full-time employees at Bellco SpA at the time this study was performed.

References


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