Comparative in vitro efficacies of ethanol-, EDTA- and levofloxacin-based catheter lock solutions on eradication of Stenotrophomonas maltophilia biofilms

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The aim of this study was to compare the in vitro activity of ethanol, EDTA and levofloxacin (Levo), alone or in combination, on biofilms of Stenotrophomonas maltophilia recovered from patients with catheter-related bloodstream infections (CRBSIs) at a university hospital in Argentina. First, 24 and 48 h biofilms were formed in microtitre plates and challenged with 25 or 40 % ethanol for 1 h. Biofilms, of the 14 local isolates and from the reference strain K279a, were eradicated after both treatments as shown by plate counts and the regrowth technique. Second, 24 h biofilms of all isolates were established in silicone catheter segments and challenged with 25 or 40 % ethanol, Levo (2.5 mg ml−1), EDTA (30 mg ml−1), 25 % ethanol–EDTA or Levo–EDTA for 1, 3 and 24 h. Viable counts of biofilms treated for 1 h with 25 or 40 % ethanol or 25 % ethanol–EDTA were under the limit of detection. Killing of biofilms by Levo or Levo–EDTA was gradual and it was only after 24 h of treatment that no differences could be seen between the effects of these catheter lock solutions (CLSs) and those of ethanol (P＞0.05). Levo–EDTA, in combination, did not act synergistically against biofilms. After 24 h of exposure, EDTA did not eradicate biofilms but reduced biofilm survival rates to 1–5 %. The effect of the different CLSs on biomass reduction, estimated by crystal violet staining, was highly dependent on the isolate, and the most effective agents were 25 and 40 % ethanol. Our results suggest that when used as a CLS for short periods, ethanol at low concentrations, alone or in combination with a chelator, can decontaminate the line from S. maltophilia in cases of CRBSI and help, in conjunction with systemic antibiotics, in the retention of precious vascular catheters.

INTRODUCTION

Stenotrophomonas maltophilia is a widespread environmental bacterium that has become a nosocomial pathogen of increasing importance. In fact, it is the third most common nosocomial non-fermenting Gram-negative bacterium (Crossman et al., 2008). Infection occurs principally in immunocompromised subjects and in patients exposed to invasive devices. S. maltophilia can cause catheter-related bloodstream infections (CRBSIs), a common and major complication encountered in catheter-dependent haemodialysis patients, as well as in patients requiring long-term indwelling central venous catheters (CVCs) for antibiotic therapy, chemotherapy and parenteral nutrition (Denton & Kerr, 1998). Biofilm formation on catheters is a key element in the development of CRBSIs, which cause significant morbidity and mortality, as well as excess length of stay and cost of hospitalization (Donlan, 2011).

With its multidrug-resistant phenotype and its ability to form biofilms, the management of S. maltophilia infections presents a challenge. Biofilms exhibit phenotypic characteristics that are distinct from those of planktonic organisms, including increased resistance to host immune defences and antimicrobial compounds. Since fluoroquinolones kill non-growing bacteria and can penetrate biofilms, they represent potential agents against sessile bacteria (Donlan, 2011). In a previous report we studied the susceptibility of S. maltophilia

Abbreviations: ALT, antibiotic lock therapy; CLS, catheter lock solution; CRBSI, catheter-related bloodstream infection; CV, Crystal Violet; CVC, central venous catheter; ELT, ethanol lock therapy; Levo, levofloxacin.
recovered from device-associated infections at a university hospital in Argentina (Passerini de Rossi et al., 2009). Of the 32 isolates from this study, 84.38% were sensitive to levofloxacin and 43.75% were sensitive to ciprofloxacin. In contrast, isolates sensitive to fluoroquinolones according to their MICs, were highly resistant when growing as biofilms according to their minimum regrowth concentration (MRC) values (256–1024 mg l⁻¹). Thus, fluoroquinolone concentrations achievable during therapy cannot eradicate S. maltophilia biofilms. Nevertheless, these agents, particularly levofloxacin, could be used for antibiotic lock therapy (ALT), which involves the instillation of a concentrated antimicrobial solution, 100 to 1000 times the MIC of the planktonic bacteria, into a catheter (Mermel et al., 2009; Vanholder et al., 2010). Since catheter-related thrombosis is a risk, which commonly prompts catheter removal and can therefore compromise therapy, many studies of ALT included a combination of an antibiotic and heparin.

CRBSIs are often difficult to treat as systemic antibiotics that are generally effective in eliminating circulating bacteria frequently fail to decontaminate the line; therefore, removal of the infected CVC is required. The Infectious Diseases Society of America (IDSA) as well as the European Renal Best Practice (ERBP) guidelines suggest the use of ALT as a therapeutic option in conjunction with systemic antibiotics for CRBSIs when catheter removal is not a favourable option (Mermel et al., 2009; Vanholder et al., 2010). Catheter salvage rate is highly dependent on the infecting organism. Recent studies in which ALT and systemic antibiotics were used to treat Gram-negative rod CRBSIs have found high success rates (Fernandez-Hidalgo et al., 2006; Poole et al., 2004). However, one concern about ALT is the selection of antibiotic-resistant organisms (Vanholder et al., 2010). Alternative non-antibiotic–anti-microbial agents may be preferable to control CRBSIs without selecting for resistant pathogens. Ethanol is an antiseptic that demonstrates bactericidal and fungicidal activity against a broad range of Gram-positive and Gram-negative bacteria and fungi. Furthermore, ethanol has been found to be effective against biofilms (Chambers et al., 2006; Raad et al., 2007). A recent review summarized available data regarding ethanol lock therapy (ELT), including studies differing in their use of ethanol concentrations (25–70%), luminal dwell times (4–24 h), inclusion of anticoagulants, use of systemic antibiotics and techniques for prevention or treatment of CRBSIs (Maiefski et al., 2009). Although the data are limited, ELT seems to be a promising option for the treatment and prevention of CRBSIs in adult and paediatric patients (Sherertz et al., 2006; Vanholder et al., 2010). Another promising candidate for use in catheter lock solutions (CLSs) is the metal chelator EDTA because of its combination of antibiofilm activity and anticoagulant activity (Bleyer et al., 2005). Raad et al. (2007) reported that a minocycline-EDTA solution in 25% ethanol eradicated methicillin-resistant Staphylococcus aureus and Candida parapsilosis biofilms grown on catheter discs after an exposure time of 1 h.

The aim of this work was to compare the in vitro activity of ethanol, EDTA and levofloxacin (Levo), alone or in combination, on pre-formed biofilms of S. maltophilia recovered from patients with CRBSIs at a university hospital in Argentina.

**METHODS**

**Bacterial isolates.** S. maltophilia isolates were recovered from 14 patients with device-associated bacteraemia (10 with vascular catheters and four with haemodialysis catheters) between January 2006 and April 2010 at Hospital de Clínicas José de San Martin, Buenos Aires, Argentina. Isolates were identified as S. maltophilia by using an API 20NE kit (bioMérieux) and frozen at −20°C. S. maltophilia K279a, isolated from the blood of an oncologic patient, was used as a reference strain (Crossman et al., 2008).

**Antimicrobial agents.** Biofilms were tested against the following CLS prepared in sterile distilled water: 25 or 40% (v/v) ethanol (Merck), Levo (Tavanic) at 2.5 mg ml⁻¹, EDTA disodium salt dihydrate (Sigma–Aldrich) at 30 mg ml⁻¹, 25% ethanol plus EDTA, and Levo plus EDTA. Sterile normal saline (NS; 0.9%, w/v, NaCl) served as the control solution.

**Biofilm formation in microtitre plates.** To establish a biofilm, overnight cultures of S. maltophilia in tryptone soya broth (TSB, Oxoid) were standardized to contain approximately 10⁶ c.f.u. ml⁻¹ (Passerini de Rossi et al., 2009). For each test condition, eight wells of a sterile flat-bottom 96-well polystyrene microtitre plate (TPP) were filled with 100 μl volumes of the standardized inoculum. Uninfected medium controls were included. After incubation at 35°C for 24 h, the culture medium was removed from each well and plates were washed twice with sterile NS to remove non-adherent cells. Biofilms were then treated with different CLSs.

**Biofilm formation in silicone catheter segments.** Silicone catheters (Silmag) were cut into segments 2 cm in length under sterile conditions and incubated stationary at 35°C in 4 ml TSB containing −1×10⁶ c.f.u. ml⁻¹ each S. maltophilia isolate (Passerini de Rossi et al., 2007). After 24 h of incubation, catheter segments were removed, washed with NS to eliminate non-adherent bacteria and placed in tubes containing 2 ml different CLSs or NS as a control.

**Effect of different CLSs on 24 h-biofilm viability.** S. maltophilia local isolate and strain K279a biofilms formed in microtitre plate wells were challenged with 25 and 40% ethanol. For each isolate, eight wells were treated with 120 μl each agent, and eight control wells were treated with 120 μl sterile NS. After 1 h at 35°C, the agents were removed and all wells were washed three times with NS. The effect of the treatments was evaluated by the regrowth assay and by plate counts (Passerini de Rossi et al., 2009). The regrowth of sessile cells was determined by adding 120 μl fresh TSB to each well. A positive result was recorded if any of the eight wells showed growth after 48 h of incubation at 35°C. The viability of the biofilms was also determined by plate counts. The surfaces of the wells were scraped with sterile cotton swabs (four wells per treatment). The swabs were transferred into tubes containing 2 ml NS, sonicated (ELMA Transonic T1-H-5) for 3 min and vortexed vigorously to aid dissolution of bacterial clumps. The number of viable cells was estimated by plating 10-fold serial dilutions of the suspensions on tryptone soya agar (TSA, Oxoid). After 24 h of incubation at 35°C colonies were enumerated and recorded as the mean c.f.u. ml⁻¹.

Biofilms from all S. maltophilia isolates established in silicone catheter segments were exposed to either 2 ml NS, 25% ethanol, 40% ethanol,
Levo, EDTA, 25 % ethanol–EDTA or Levo–EDTA. Four catheter segments were challenged with the same solution for 1, 3 and 24 h at 35 °C. After the prescribed contact time, the catheter segments were removed, washed twice in NS, transferred into tubes containing 2 ml NS, sonicated, vortexed and plated in serial dilutions as described previously to determine the number of viable cells. C.f.u. were counted and converted to log10 c.f.u. per catheter segment. The limit of detection in our experimental conditions was 40 c.f.u. per catheter segment. The percentages of survival after treatment with different CLSs were calculated by the ratio between the values of c.f.u. per catheter segment with and without CLSs.

**Effect of different CLSs on 24 h-biofilm biomass.** Biofilms from all S. maltophilia isolates formed in microtitre plate wells were challenged with each CLS for 1, 3 and 24 h. After the corresponding dwell time, the agents were removed and the wells were washed and stained with 0.01 % (w/v) Crystal Violet (CV) for 30 min. The stained biofilms were washed and extracted with 95 % (v/v) ethanol. The amount of biofilm was quantified by measuring the OD590 of dissolved CV using a Multiskan EX plate reader (Thermo electron corporation). The percentages of biomass after treatment with different CLSs (eight wells per agent) were calculated by the ratio between the OD590 values with and without the agent (Passerini de Rossi et al., 2009).

**Statistical analysis.** All assays were carried out at least in quadruplicate and repeated three times. Results were determined by two-way analysis of variance (ANOVA), followed by Bonferroni’s multiple-comparison test (GraphPad Prism version 5 for Windows, GraphPad Software). Differences were considered significant at P-values <0.05.

**RESULTS AND DISCUSSION**

Intravascular catheters are highly used in modern medicine and unfortunately can provide access to bacteria. During the period of January 2006 to April 2010, S. maltophilia was isolated from 14 patients with CRBSIs at a university hospital in Argentina. All S. maltophilia local isolates were able to form biofilms, a key factor in the development of CRBSIs. In this study we compared the **in vitro** activity of different CLSs on S. maltophilia pre-formed biofilms.

**Effect of ethanol on the viability of biofilms formed in microtitre plate wells**

Using a static multiwell incubation model, we found that 24 h biofilms of the 14 S. maltophilia local isolates and strain K279a were completely eradicated within 1 h of exposure to 25 or 40 % ethanol. Both treatments resulted in an absence of regrowth of sessile cells after 48 h of incubation at 35 °C in TSB, indicating that all sessile cells present in the biofilms were killed by ethanol. As expected, regrowth was observed for biofilms of all isolates after control treatment with NS. The same results were observed when treating 48 h biofilms with these low ethanol concentrations (data not shown). These results were confirmed by plate counts since viable bacteria were not detected after ethanol treatment. Ethanol at low concentrations completely eradicated the 24 h S. maltophilia biofilms, which initially contained $2.0 \times 10^5$–$4.5 \times 10^5$ c.f.u. ml$^{-1}$, depending on the isolate. Our findings are in agreement with a recent study by Qu et al. (2009), which found that exposure of coagulase-negative staphylococcal biofilms to 40 % ethanol for 1 h was sufficient to eradicate the biofilm bacteria.

**Effect of different CLSs on the viability of biofilms formed in catheter segments**

The effectiveness of 25 or 40 % ethanol, Levo, EDTA, 25 % ethanol–EDTA or Levo–EDTA was assessed by using an **in vitro** model of 24 h biofilms formed on silicone catheter segments. Performance of each CLS against S. maltophilia strain Sm13 biofilms at different contact times is summarized in Fig. 1. Strain Sm13, isolated from a patient with vascular catheter-related bacteraemia, was the strongest biofilm producer among the local isolates according to CV staining (OD590 2.100). All treatments resulted in a significant reduction in number of viable sessile cells compared to the control, NS, at the three contact times (P<0.001). The control silicone catheter segments were heavily colonized with mean viable cell counts of $1.36 \times 10^6$, $1.04 \times 10^6$ and $1.80 \times 10^6$ c.f.u. per catheter segment at 1, 3 and 24 h, respectively.

Viable cell counts of biofilms treated for 1 h with 25 or 40 % ethanol or 25 % ethanol–EDTA were under the limit of detection. It is of note that ethanol, alone or in
combination with EDTA, was the only agent successful in rapidly eradicating sessile cells from Sm13 biofilms. In contrast, significant but limited decreases in c.f.u. per catheter segment counts were observed when treating Sm13 biofilms with EDTA, Levo or Levo–EDTA for 1 h. At the 3 h time point, a log₁₀ factor reduction of 2.59 or 2.16 of viable cells was observed after exposure to Levo or Levo–EDTA, respectively; a significantly greater reduction compared with the effect of using EDTA alone (0.87) (P< 0.001). Killing of biofilms by Levo or Levo–EDTA was gradual, and only at 24 h of treatment did the ANOVA–Bonferroni’s multiple-comparison test determine that no differences existed among these CLSs and ethanol (P>0.05). There was no significant difference in mean log₁₀ c.f.u. per catheter segment reductions between Levo and Levo–EDTA at the three contact times (P>0.05), indicating that the two compounds in combination Levo–EDTA did not act synergistically against S. maltophilia biofilms. On the other hand, treatment with EDTA for 24 h only reduced the number of biofilm bacteria by a factor of 10².

A similar CLS performance was observed against all S. maltophilia isolates with some differences in reduction of biofilm viability by Levo and Levo–EDTA after 1 and 3 h of treatment, and by EDTA alone. Table 1 shows the percentage of survival of strain Sm13, Sm14 and Sm18 biofilms after different exposure times to these agents. Strains Sm14 and Sm18, isolated from patients with haemodialysis-catheter-related bacteraemia, were the isolates with the highest percentages of survival after EDTA treatment. For strain Sm18, the percentages of survival were 16 and 17 % after 1 and 3 h of exposure, respectively, and for Sm14 the percentage of survival was 3.9 % after 24 h-challenge. With respect to Levo and Levo–EDTA, strain Sm14 was the most sensitive isolate with percentages of survival <0.1 % after 3 h of exposure.

These results are in agreement with those of Sherertz et al. (2006) and Qu et al. (2009), and support the use of ethanol as a CLS at low concentrations for a short dwell time, instead of antibiotics at high concentrations for a long period to treat CRBSIs.

EDTA (30 mg ml⁻¹) in combination with minocycline was used successfully as a CLS in clinical studies and no cases of toxicity have been reported (Bleyer et al., 2005). In our study, the same concentration of EDTA produced reductions in the number of S. maltophilia viable sessile cells but failed to eradicate biofilms, and the combination of Levo–EDTA did not have a synergistic effect. However, EDTA in combination with 25 % ethanol achieved rapid biofilm eradication. In addition, ethanol is compatible with EDTA and citrate, two anticoagulant agents proposed to be used in CLSs in order to circumvent the need for heparin and the potential complications that arise from its use (Bleyer et al., 2005; Vanholder et al., 2010).

**Effect of different CLSs on biofilm biomass**

All S. maltophilia local isolates were able to form biofilms; however, CV staining showed differences in the ability to form biomass among isolates (OD₅₄₀ 0.750–2.100). Spectrophotometric analysis of S. maltophilia biofilms stained with CV revealed that after 1 h of treatment 25 or 40 % ethanol reduced biomass by 30–75 %, depending on the isolate. A similar range of biomass reduction was observed after a 24 h-challenge with Levo (35–70 %). On the other hand, after the 24 h-challenge, EDTA reduced the biofilm biomass of four isolates by 20–25 %, enhanced the biomass production of three isolates by 15–30 % and did not affect biofilm biomass production of seven isolates. Furthermore, in the presence of EDTA, 25 % ethanol and Levo caused a lower reduction of biomass (20–45 %).

<table>
<thead>
<tr>
<th>Agent</th>
<th>Exposure time (h)</th>
<th>Sm13</th>
<th>Sm14</th>
<th>Sm18</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>1</td>
<td>6.22 (3.76)</td>
<td>10.54 (3.40)</td>
<td>16.00 (5.47)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>12.04 (6.71)</td>
<td>5.41 (1.39)</td>
<td>17.00 (5.21)</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>1.07 (0.25)</td>
<td>3.90 (0.80)</td>
<td>1.50 (0.31)</td>
</tr>
<tr>
<td>Levo</td>
<td>1</td>
<td>2.13 (0.93)</td>
<td>0.47 (0.14)</td>
<td>0.91 (0.45)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.26 (0.11)</td>
<td>0.04 (0.01)</td>
<td>0.22 (0.09)</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Levo–EDTA</td>
<td>1</td>
<td>1.53 (0.90)</td>
<td>0.41 (0.15)</td>
<td>0.74 (0.26)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.88 (0.79)</td>
<td>0.05 (0.01)</td>
<td>0.39 (0.17)</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

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*Percentage biofilm survival after treatment with different CLSs was calculated by the ratio between the mean c.f.u. per catheter segment with and without the agent. Results are from three independent assays. The absolute values corresponding to 100 % at 1, 3 and 24 h, respectively, were: 1.36 x 10⁶, 1.04 x 10⁶ and 1.80 x 10⁶ c.f.u. per catheter segment for Sm13; 1.26 x 10⁶, 1.19 x 10⁶ and 1.60 x 10⁶ c.f.u. per catheter segment for Sm14; and 2.61 x 10⁶, 2.54 x 10⁶ and 2.08 x 10⁶ c.f.u. per catheter segment for Sm18.*
The effect of the different CLSs on biofilm viability was similar among the studied isolates, but the effect on biomass reduction was highly dependent on the isolate. It is important to remark that there was no relation between the ability of the CLSs to reduce biofilm biomass and their ability to kill biofilm cells.

In this study, ethanol was found to be superior to the other CLSs in the eradication of *S. maltophilia* biofilms. A major advantage of ethanol as a CLS would be the broad-spectrum activity against planktonic and sessile multidrug-resistant Gram-positive and Gram-negative bacteria, as well as fungi, based on protein denaturation, which makes resistant Gram-positive and Gram-negative bacteria, as spectrum activity against planktonic and sessile multidrug-resistant *S. maltophilia*. The complete genome, comparative and functional analysis of Stenotrophomonas maltophilia reveals an organism heavily shielded by drug resistance determinants. *Genome Biol* 9, R74.


ACKNOWLEDGEMENTS

The authors thank Maxwell Dow, Biomerit Research Centre, Department of Microbiology, Biosciences Institute, National University of Ireland, Cork, Ireland, for providing the *S. maltophilia* K279a strain. This research was supported by grants from UBACyT, Argentina (grant nos B117 and B084).

REFERENCES


