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ARTICLE

Activity of ethanol and daptomycin lock on biofilm generated by an in vitro dynamic model using real subcutaneous injection ports

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Abstract Vancomycin lock solution (LS) is recommended for the conservative treatment of subcutaneous injection port (SIP)-related infections, but may be associated with failure. We used an in vitro dynamic model of biofilm formation in an SIP, based on a continuous flow circulating via a real SIP, to assess the effectiveness of vancomycin (5 mg/ml), daptomycin (5 mg/ml) and ethanol 40 % LS in eradicating a pre-established *Staphylococcus epidermidis* biofilm. Heparin, Ringer's lactate and enoxaparin sodium LS were used as controls. The logarithmic reductions of colony-forming units (CFU) were compared by Student's *t*-test. After 24 h of exposure, the vancomycin LS did not exert a greater bactericidal effect than the heparin LS control (mean logarithmic reduction: 2.27±0.58 vs. 1.34±0.22, respectively, $p=0.3$). The mean logarithmic reduction was greater with daptomycin LS (5.45±0.14 vs. 0.39±0.12, $p<0.01$) and ethanol LS (6.79±1.03 vs. 1.43±0.54, $p=0.02$). Bacterial revival after exposure to 24 h of LS was assessed. The mean viable bacteria count was significantly higher for vancomycin LS (9.36±0.10 $\log_{10}$CFU) and daptomycin LS (9.16±0.02 $\log_{10}$CFU) than for ethanol LS (2.95± 1.65 $\log_{10}$CFU). Ethanol appeared to be the most attractive option to treat SIP-related infection, but its poor ability to entirely disrupt the biofilm structure may require its use in association with a dispersal agent to avoid renewal of the biofilm.

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Introduction

Subcutaneous injection ports (SIPs) are composed of a subcutaneously inserted injection port made of titanium or plastic that is linked to a central venous catheter made of silicone rubber or polyurethane [1]. They can be used to take blood samples and to administer drugs, blood products or parenteral nutrition [1]. The main advantages over external central venous catheters include a better quality of life for the patient owing to unrestricted mobility and reduced susceptibility to infection [2]. SIPs are used worldwide, mainly in the treatment of patients with cancer or chronic diseases requiring permanent central venous access.

SIP-related infections are less frequent than with other central venous lines, having an estimated incidence of 0.016 to 1 infections per 1,000 device days [3]. The main microorganism isolated from infected SIPs is coagulase-negative staphylococci (CoNS), especially in patients receiving long-term parenteral nutrition [4, 5]. However, Gram-negative bacteria, *Staphylococcus aureus*, fungi, enterococci or any other bacteria may also be involved [5]. The rate of SIP-related infection is higher in terminally ill patients with advanced cancer and in paediatric haematology–oncology patients [6, 7].

There are two options for the management of non-complicated SIP-related infections due to CoNS: removal or salvage therapy with retention of the device. When the device is left in place, SIP CoNS-related infections are more difficult to sterilise than with other external central venous catheters and are associated with a greater likelihood of recurrent bacteraemia [6, 8, 9]. This may be due to the ability of CoNS to produce a biofilm formed by bacteria in stationary phase embedded in an extracellular matrix. The biofilm protects bacteria against the immune system and antibiotics, and constitutes a depository that may release bacteria into the bloodstream [10]. It is thought

to be the leading cause of the persistence of CoNS infection and of recurrent CoNS bacteraemia [8, 9].

Retention should be combined with antibiotic-lock therapy and, in cases of bloodstream infection, with systemic antibiotic therapy [11]. The antibiotic-lock technique consists of filling the chamber and the catheter with an antimicrobial agent at a concentration high enough to reach 100 to 1,000 times its minimal inhibitory concentration (MIC). When the SIP is not used, the solution dwells for a given period of time according to the characteristics of the antimicrobials (generally 24 h) [12–14]. For CoNS SIP-related infections, the antibiotic lock solution (LS) recommended is vancomycin, at a concentration of 5 mg/ml, for 7–14 days, with renewal of the solution every 24–48 h [11]. Vancomycin LS reaches very high concentrations, 500 to 1,000 times greater than the MIC, and is considered to be effective in killing sessile bacteria embedded in the biofilm [11]. However, when the device is left in place, SIP-related infections are more difficult to sterilise than with other external central venous catheters and are associated with a greater likelihood of recurrent bacteraemia, especially with CoNS [2, 8, 9, 15].

The ability of vancomycin to eradicate *S. epidermidis* biofilm has been studied in several in vitro models of experimental catheter-related infection, but with conflicting results. Some authors suggested that vancomycin, even at high doses, is unable to eradicate bacterial colonisation from catheters [16, 17], while others found that vancomycin is effective in eradicating *S. epidermidis* biofilm [18, 19]. There is only one randomised placebo-controlled trial that has compared lock therapy (mainly with vancomycin) and placebo, in 40 patients with SIP-related infections [20]. The authors reported a failure rate of 33 % in the lock therapy group, with 3 patients out of 21 relapsing during follow-up [20]. Retrospective studies confirmed the potential interest of ALT, but the real effectiveness and the length of ALT treatment remain uncertain [4, 21].

Because the regular increase in vancomycin MICs among CoNS isolates will make this antibiotic less effective against such bacteria in the near future, new ALT compounds with greater and swifter effectiveness should be studied in order to reduce the length of ALT treatment, the risk of antimicrobial resistance and the rate of failure [19, 22–24]. In this study, we used a dynamic model of SIP-related infection adapted from a previously described method [23] to assess the activity of vancomycin, daptomycin and ethanol on a pre-established CoNS biofilm in an SIP.

Materials and methods

Microorganism

An *S. epidermidis* (CIP 21.25, Collection Institut Pasteur, Paris, France) biofilm was produced over 72 h inside an SIP (port made of silicon rubber with an inner volume of 0.6 ml and a polyurethane catheter 61 cm long with an inner diameter of 1.6 mm, X-port *isp*, Bard Access Systems, Salt Lake City, UT, USA). *S. epidermidis* 21.25 exhibits decreased susceptibility to teicoplanin (MIC: 8 mg/L) but remains susceptible to daptomycin (MIC: 0.38 mg/L) and vancomycin (MIC: 2 mg/L).

Method of biofilm formation

Our model of biofilm production was adapted to the SIP from an experimental model described for endoscopes [25, 26]. The SIP was aseptically connected to sterile polyvinylchloride (PVC) tubes (Nalgène, Illkirch, France) to form a loop that was supplied with tryptone soya broth culture medium (TSB, CM0129, Oxoid, Cambridge, United Kingdom). The system was activated by two pumps (Watson Marlow 205S, La Queue Lez Yvelines, France): one provided a continuous flow of TSB medium in the system and the other a homogeneous diffusion of the TSB medium and of the *S. epidermidis* suspension in the loop (Fig. 1). Pump 1 was turned on at a speed of 23 ml/min to fill the system with TSB medium and then stopped. The circuit was inoculated at one time point with 20 ml of a suspension containing about 10^8 *S. epidermidis* per ml. Pump 2 was turned on at a speed of 15 ml/min for 4 h to allow the dissemination and the first adhesion of bacteria in the loop. The continuous flow was then turned on at a speed of 2 ml/min and the speed of pump 2 was increased to 23 ml/min. The system was run for 72 h to allow biofilm formation.

Lock solutions

Vancomycin LS was composed of vancomycin at a concentration of 5 mg/ml (Vancocin® 250 mg, Mylan®, Saint Priest, France) and heparin at a concentration of 2,500 IU/ml (Héparine Choay® 25,000 IU/5 mL, Sanofi-Aventis, Paris, France)

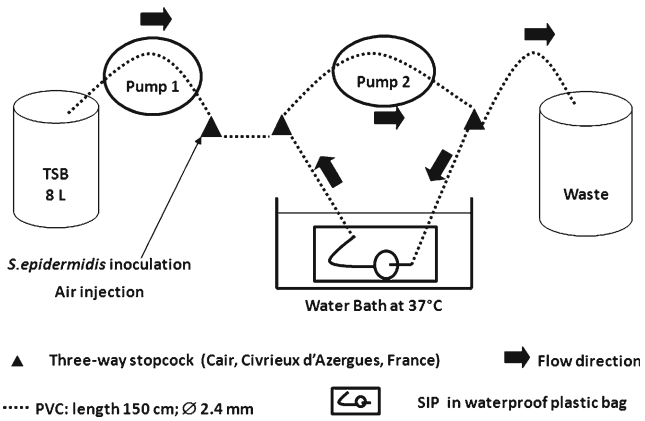

Fig. 1 Model of biofilm formation in the subcutaneous injection port (SIP)

Table 1 Mean bacterial count recovered in the subcutaneous injection port (SIP) after antimicrobial lock. The quantity of biofilm present in the SIP was given in each trial as colony-forming units (CFU) per SIP

Mean bacterial count (CFU/SIP)	Control SIP	After antimicrobial LS	After control LS
Vancomycin trial	5.06E+08	3.44E+06	1.96E+07
Daptomycin trial	1.14E+08	4.80E+02	2.75E+08
Ethanol trial	1.51E+09	1.92E+03	4.02E+07

LS lock solution

diluted in 0.9 % NaCl (Versylène®, Fresenius, Sèvres, France) [11]. Daptomycin LS was composed of daptomycin at a concentration of 5 mg/ml (Cubicin® 500 mg, Novartis, Horsham, United Kingdom) diluted in Ringer's lactate (Macoflex® 250 ml, MacoPharma, Mouveaux, France, containing 80 μg/ml calcium [Ca^{2+}]) [19, 22]. Ethanol LS was composed of ethanol 40 % and enoxaparin sodium at a concentration of 400 IU/ml (Lovenox® 4,000 IU anti-Xa/0, 4 ml, Sanofi-Aventis, Paris, France) diluted in 0.9 % NaCl. Enoxaparin is an anticoagulant, which mainly inhibits factor Xa by activating antithrombin III. Thus, enoxaparin decreases thrombin formation and, ultimately, prevents clot formation. This combination of ethanol/enoxaparin showed stable antithrombotic and antimicrobial activities and led to no structural degradation of the catheter surfaces (patent submission number: 1000136848; unpublished data). Control LS were heparin sodium at a concentration of 2,500 IU/ml and Ringer's lactate and enoxaparin sodium at a concentration of 400 IU/ml. Three millilitres of each LS were instilled into the injection port to fill up the SIP. The catheter was then clamped, placed in a sterile drape and incubated at 37 °C for 24 h. In a first set of experiments, we assessed the activity of the three LS (vancomycin, daptomycin and ethanol) dwelled for 24 h on a 72-h bacterial biofilm. Three SIPs were tested in each trial: one used as a control, one filled with the antimicrobial LS (antibiotic or ethanol) and one filled with the control LS. Each trial was performed in triplicate. In a second set of experiments, we assessed the ability of the bacteria to reform a biofilm after 24 h of exposure to antimicrobial LS. The treated SIPs were reconnected to the system for 72 h. No additional bacterial inoculum was added to the system. Each trial was performed in triplicate.

Recovery of treated *S. epidermidis* from the SIP

The viable bacteria in LS were recovered under a laminar flow hood without centrifugation. The injection port and the catheter were then separated and processed separately. The treated biofilm was recovered by a previously described mechanical technique [25, 26]. The bacteria were recovered from the injection port by three successive flushes of 3 ml of Letheen Broth® (VWR Prolabo, Fontenay-sous-Bois, France). Between each flush, the injection port was vortexed (Vortex-Genie, shake 8, Scientific Industries Inc., Bohemia,

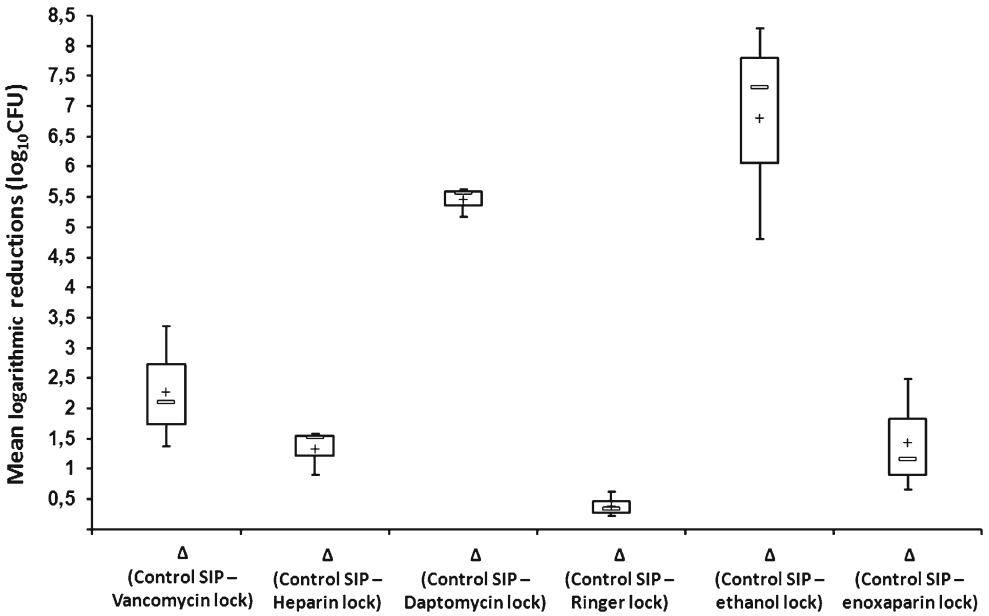

Fig. 2 Mean logarithmic reduction after antimicrobial lock. Δ: calculated difference between the SIP used as the control and the antimicrobial lock (vancomycin, daptomycin and ethanol) or the control lock (heparin, Ringer's lactate and enoxaparin sodium). Comparisons between groups were performed by Student's *t*-test with a significance level set at 0.05. *p*-value of mean logarithmic reductions: vancomycin lock solution (LS) versus heparin LS, $p=0.3$; daptomycin LS versus Ringer's lactate LS, $p<0.01$; ethanol LS versus enoxaparin LS, $p=0.02$; vancomycin LS versus daptomycin LS, $p=0.02$; vancomycin LS versus ethanol LS, $p=0.02$; daptomycin LS versus ethanol LS, $p=0.4$. The box-and-whisker plots show the mean (+), the median (horizontal bar inside the box), the 25th/75th percentile and the extreme values as bars at the extremities of the whiskers

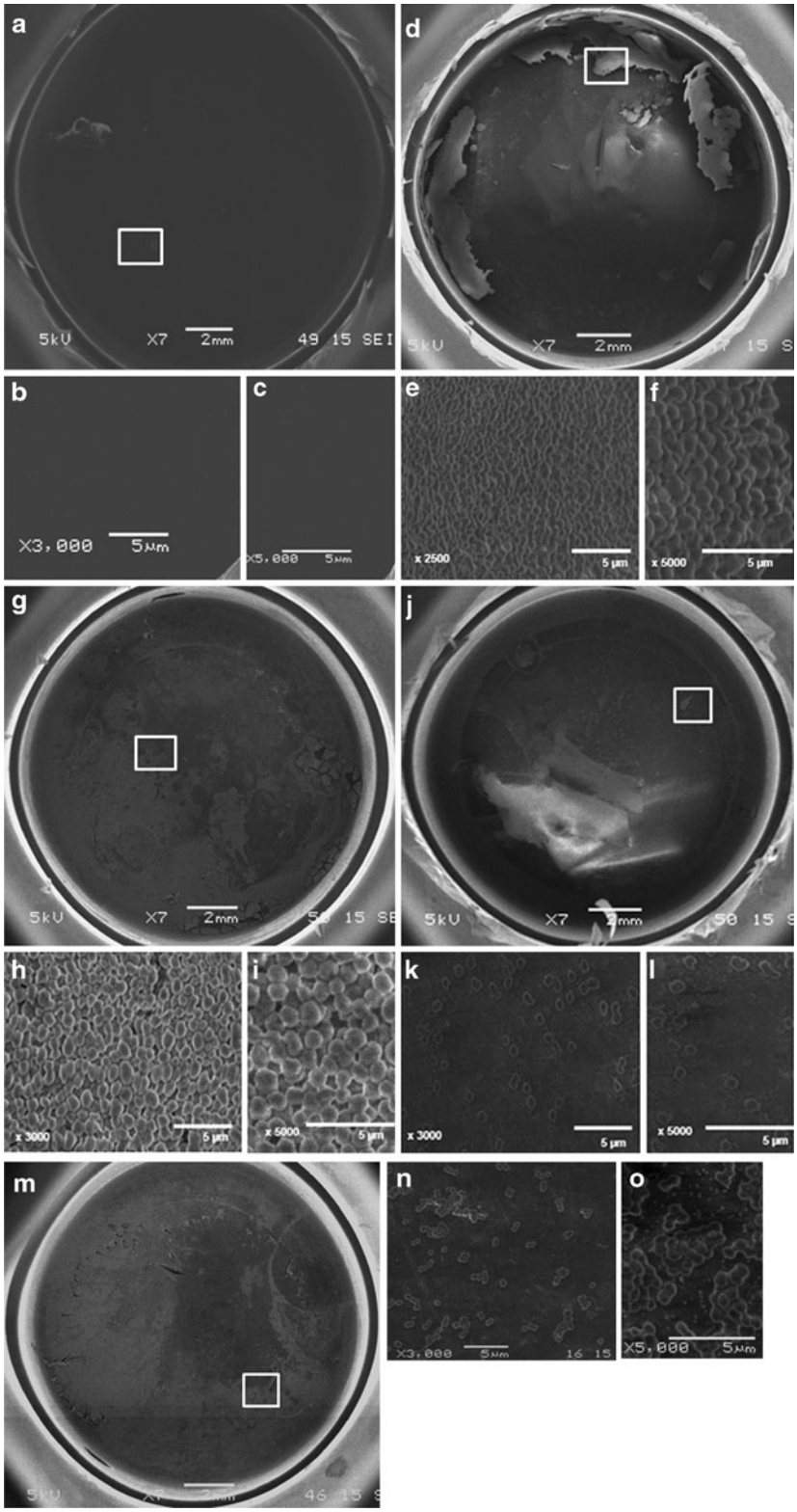

Fig. 3 Scanning electron microscopy (SEM) of the injection ports. *d* lens distance; *the white bars* represent scale; magnification: ×7, ×3,000, ×5,000; *the white square* indicates the magnified area. **a**–**c** non-infected injection port; **d**–**f** control; **g**–**i** vancomycin lock; **j**–**l** daptomycin lock; **m**–**o** ethanol lock

NY, USA) for 5 min, ultra-sonicated at 125 W (Branson 2210, Bransonic, Danbury, CT, USA) for 5 min and then vortexed again for 5 min. After removal of the septum, the injection port was immersed in 10 mL of Letheen Broth® and scraped with a scalpel before a session of vortex–sonication–vortex. Bacterial recovery from the catheter was performed by three successive flushes (10 ml, 3 ml and 3 ml) of Letheen Broth®. Between each flush, the catheter was subjected to vortex–

sonication–vortex. It was then cut into several 1-cm portions that were immersed in 10 ml of Letheen Broth® and subjected to vortex–sonication–vortex. The solutions recovered from the lock, the injection port and the catheter were diluted up to 10^{-7} and plated on Tryptone soya plates (TSA bioMérieux, Lyon, France) that were incubated at 37 °C for 24 h. The bacterial count from the lock solution was added to the bacterial count found in the catheter and the injection port. The quantity of biofilm present in the SIP or in the catheter was given in each trial as $\log_{10}$CFU (colony-forming units) per SIP or as CFU per SIP.

Scanning electron microscopy (SEM) of the injection port

Samples were fixed in 2 % glutaraldehyde in cacodylate buffer (0.2 M at pH 7.2) for 1 h. The septum was then aseptically removed and the injection port filled with the fixative for 24 h at 4 °C. Samples were mounted on a metallic support with an adhesive carbon tab and then sputter-coated with gold-palladium (JFC-1300; Jeol, Tokyo, Japan). Microscopic analysis was made using a scanning electron microscope (JSM-6060LV; Jeol) in high-vacuum mode.

Statistics

We calculated the logarithmic reduction for each trial between the SIP used as the control and the antimicrobial LS (vancomycin, daptomycin and ethanol) or the control LS (heparin, Ringer's lactate and enoxaparin sodium). Comparisons between groups were made by Student's *t*-test with a significance level set at 0.05. Statistical analysis was done with SPSS software (version 10.1 for Windows; SPSS, Inc., Chicago, IL, USA).

Results

SIP experimental biofilm model

The strain of *S. epidermidis* (CIP 21.25, Collection Institut Pasteur, Paris, France) was chosen because it showed commonly observed susceptibility results and was able to produce a biofilm on a plastic support (data not shown). The injection of bacterial inoculum at one time point was chosen to simulate the one-time contamination of the SIP by skin-related bacteria, such as that which may occur during a failure of skin antisepsis.

We then assessed the reproducibility of the biofilm formation in nine SIPs. The mean viable bacteria count was 8.35 $\log_{10}$CFU per injection port (range 7.56–9.52), 7.83 $\log_{10}$CFU per catheter (range 6.76–8.68) and 8.51 $\log_{10}$CFU per SIP (range 7.88–9.57). Low standard deviations were found for the injection port, catheter and SIP (0.19, 0.21 and 0.17, respectively). There was no significant difference between the total bacterial recovery rates in the injection port and the catheter (p=0.1).

Activity of antimicrobial LS on a 72-h-old *S. epidermidis* biofilm

Table 1 and Figure 2 show the mean viable bacteria count and the mean logarithmic reductions in CFU of 72-h-old *S. epidermidis* biofilms after a 24-h exposure to antimicrobial LS (vancomycin, daptomycin or ethanol) and to control LS (heparin in Ringer's lactate and enoxaparin sodium).

The vancomycin LS did not exert a greater bactericidal effect than its heparin control LS (mean logarithmic reduction 2.27±0.58 vs. 1.34±0.22, respectively, p=0.3). In contrast, the mean logarithmic reduction was statistically greater than that of the control for daptomycin LS (5.45±0.14 vs. 0.39±0.12, p<0.01) and ethanol LS (6.79±1.03 vs. 1.43±0.54, p=0.02). Compared to that of vancomycin LS, the mean logarithmic reduction was greater after daptomycin LS exposure (p= 0.02) and after ethanol LS exposure (p=0.02). No significant difference was found between daptomycin and ethanol LS (p=0.4).

The biofilm was well visualised by SEM of the injection ports in the control (Fig. 3d–f) and after vancomycin LS

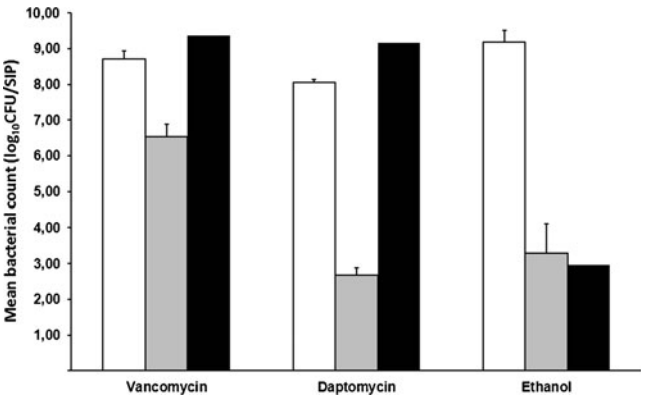

Fig. 4 Mean bacterial count in control SIP immediately after antimicrobial lock solution and bacterial revival after 24 h of exposure to antimicrobial LS followed by 72 h in the biofilm development system. SIPs exposed to an antimicrobial LS (vancomycin, daptomycin or ethanol) were reconnected to the biofilm development system for a 72-h period without additional bacterial inoculum. *White bars* bacterial count of control SIP; *grey bars* bacterial count immediately after antimicrobial lock solution; *black bars* bacterial regrowth after 72 h. Comparisons between groups after 72 h of regrowth were performed by Student's *t*-test with a significance level set at 0.05. *p*-values of the mean bacterial count of the SIPs: vancomycin LS versus daptomycin LS (p=0.13); vancomycin LS versus ethanol LS (p=0.018)

exposure (Fig. 3g–i). Despite the activity, we also observed a biofilm after treatment with daptomycin LS (Fig. 3j–l) and with ethanol LS (Fig. 3m–o). Figure 3a–c show a non-infected injection port.

Biofilm reformation after 24 h of exposure to lock solution

We assessed bacterial revival after 24 h of exposure to antimicrobial LS followed by 72 h of further incubation in our biofilm development system without additional bacterial inoculum (Fig. 4). The mean viable bacteria count was high in assays performed with vancomycin LS (9.36±0.10 $\log_{10}$CFU/SIP) and with daptomycin LS (9.16±0.02 $\log_{10}$CFU/SIP). The mean bacteria count of ethanol LS was significantly lower than that of vancomycin LS (2.95±1.65 $\log_{10}$CFU/SIP vs. 9.36±0.10 $\log_{10}$CFU/SIP, p=0.018).

Discussion

We used an in vitro dynamic model of biofilm formation in an SIP, based on a continuous flow circulating via a real SIP. The model was adapted from a previously described technique [25, 26]. It was able to study not only biofilm formation in the catheter but also in the port, which offers a large surface for the bacteria to adhere to [8, 9]. Quantification of the biofilm was determined by enumeration of the CFU, the only method suitable for the solutions recovered from the SIP. The biofilm obtained was reproducible. We used the model to assess the effectiveness of three different LS with a dwell time of 24 h to eradicate a 72-h-old, *S. epidermidis*-related biofilm adherent to the SIP. Vancomycin had very limited activity against bacteria embedded in SIP-adherent biofilm. The mean logarithmic reduction of CFU after vancomycin LS exposure was not statistically different from that of its control, allowing the biofilm to reform after discontinuation of treatment. Thus, our results show that vancomycin is poorly able to kill bacteria protected by their sessile state, despite a concentration 2,500 times greater than the MIC. This lack of efficacy is consistent with observations in certain in vitro and clinical studies, in which vancomycin LS therapy was associated with a high failure rate [16, 17, 20]. In contrast, several in vitro studies showed vancomycin ALT to be effective but with different models, which makes it difficult to compare results [18, 27]. None of these studies used a port linked to a catheter. In a rat model of staphylococcal central venous infection, Van Praagh et al. showed that 3 days of vancomycin ALT and daptomycin ALT were equally effective in eradicating *S. epidermidis* biofilms [19]. However, the authors combined systemic antibiotic therapy with ALT and no port was included in the experiment. Chauhan et al. recently developed a promising rat model of SIP-related infection but did not evaluate the effectiveness of vancomycin ALT [28]. Daptomycin is known for its ability to penetrate the biofilm [19]. In addition, it was more effective in killing bacteria than vancomycin in our model. However, despite the fact that its concentration reached 13,000 times the MIC, 24-h daptomycin LS did not completely eradicate the bacteria from the SIP. This could facilitate recurrences or favour subsequent distinct infections with other bacteria, which may use the structure of the pre-existing biofilm (mainly the matrix) to adhere to the port and catheter [9]. In addition, the wide use of daptomycin LS poses the risk of staphylococcal resistance, and daptomycin is ineffective against Gram-negative bacteria, which are also frequently responsible for SIP-related infections.

Ethanol was the most effective solution in this model and was able to kill most of the bacteria adhering to the SIP. This may explain why bacterial regrowth after ethanol exposure was weak in comparison with exposure to daptomycin and vancomycin. However, total eradication was not achieved, possibly owing to limited diffusion of the ethanol solution within the biofilm [29]. In addition, low ethanol concentration may promote biofilm formation [30]. Thus, although ethanol LS seems to be the most effective LS in our model, failure or distinct infections following ethanol lock treatment may occur. In future studies, another agent that is able to disperse the biofilm could be added to the classical treatment, which combines LS with systemic antibiotic therapy. Several candidates for dispersal solutions have recently emerged and could be tested, concomitantly or sequentially, with the antimicrobial lock solution [10, 31].

Compared to in vivo studies of SIP-related infections, our model has certain limits. In vivo SIPs are submitted to multiple factors such as fibrin and coagulation debris that may favour the establishment of the biofilm and its persistence [8]. The model does not take into account external infections. Lastly, the dwelling time in our study was only 24 h instead of 7–14 days, as is standard in clinical practice (the LS being renewed every 24–48 h) [11]. We chose 24 h because more manipulations of the SIP, even under a laminar hood, would have run the risk of contamination. We also believe that, in the future, LS should aim to be rapidly effective, for the convenience of patients, to reduce costs and to limit manipulation of the SIP.

In conclusion, vancomycin LS, which is recommended for the conservative treatment of SIP, was poorly effective in eradicating *S. epidermidis* biofilm in our model. Ethanol and daptomycin lock solutions were significantly more effective, but daptomycin was unable to inhibit biofilm reformation after the discontinuation of lock treatment. Ethanol appeared to be the most attractive option to treat SIP-related infection.

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Conflict of interest The authors declare that they have no conflict of interest.

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