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In vitro activity of EDTA or L-arginine in combination with gentamicin, vancomycin or amikacin as lock therapy against a wide spectrum of biofilm-forming clinical strains isolated from catheter-related infections

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Short title: gentamicin-EDTA against Gram-positive and Gram-negative clinical strains

Key words: Biofilm; persisters; aminoglycoside; antibiotic lock therapy, adjuvant strategy, *in vitro* model

Abstract

Objectives. Treatment of catheter-related bloodstream infections (CRBSI) is hampered by the characteristic tolerance of bacterial biofilms towards antibiotics. Our objective was to study the effect of the combination of antibiotics and the alkaline amino acid L-arginine or the cation chelator EDTA on the bacterial mortality of *in vitro* biofilms formed by an array of clinical strains responsible for CRBSI and representative of epidemiologically relevant bacterial species.

Methods. Among 32 strains described in a previous clinical study, we focused on the most antibiotic-tolerant strains including coagulase-negative staphylococci (n=4), *Staphylococcus aureus* (n=4), *Enterococcus faecalis* (n=2), *Pseudomonas aeruginosa* (n=4) and Enterobacteriaceae (n=4). We used *in vitro* biofilm model (96-well plate assay) to study biofilm tolerance and we tested various combinations of antibiotics and non-antibiotic adjuvants. Gentamicin, amikacin or vancomycin were combined with disodium EDTA or L-arginine during 24 hours, to reproduce the Antibiotic Lock Therapy (ALT) approach. Mortality of biofilm bacteria was measured by cfu quantification after a vigorous step of pipetting up and down in order to detach all biofilm bacteria from the surface of the wells.

Results. Both of our adjuvant strategies significantly increased the effect of antibiotics against biofilms formed by Gram-positive and Gram-negative bacterial pathogens. The combination of gentamicin + EDTA was active against all tested strain but one *P. aeruginosa*. The combination of gentamicin + L-arginine was active against most of tested strains with the notable exception of coagulase-negative staphylococci for which no potentiation was observed. We also demonstrated that combination using amikacin + EDTA was active against Gram-negative bacteria and vancomycin + EDTA against Gram-positive bacteria.

Conclusion. The addition of EDTA enhanced activity of gentamicin, amikacin, and vancomycin against biofilms formed by a wide spectrum of bacterial strains responsible for CRBSI.

Introduction

Following an initial report in 1988[1], several studies demonstrated that antibiotic lock therapy (ALT) could be a therapeutic option in case of catheter-related bloodstream infection (CRBSI).[1-3] ALT relies on the instillation of a small volume of highly concentrated antibiotic solution that dwells in the lumen of the catheter for 12 to 72 hours, in order to eradicate biofilm formed on the inner surface of the device.[1,4] Indeed, most of treatment difficulties encountered during CRBSI are related to the presence of high cell density bacterial communities called bacterial biofilms.[5] Biofilms display characteristic properties, including high tolerance towards antimicrobials that is defined by the ability of a subset of bacteria to survive in the presence of high concentration of antibiotics.[6,7]

Recent IDSA guidelines recommend that ALT should be used in case of conservative treatment of uncomplicated long-term intravenous catheter-related bloodstream infections caused by coagulase-negative staphylococci or Enterobacteriaceae.[8] This statement is based on different studies reporting salvage rates ranging from 80 to 90% in these situations.[4] However, other groups reported higher failure rates, even in case of coagulase-negative staphylococci infections.[9,10] Furthermore, infections caused by *Staphylococcus aureus* or *Pseudomonas aeruginosa* are still considered to be at higher risk of treatment failure, despite recent encouraging results, for the latter case.[4,11] Lastly, ALT requires locking the long-term intravenous catheter and thus reduces it's availability for 7 to 14 days. Thus, there is a dire need for more efficient locks in order to improve biofilm eradication and reduce the time during which the catheter is unavailable.

We previously demonstrated that 2 adjuvant strategies could be used to eradicate *in vivo* biofilms formed by various Gram-positive as well as Gram-negative nosocomial pathogens.[12,13] The first strategy relies on the use of EDTA, a cation chelator that destabilizes the biofilm matrix.[14] EDTA has been shown to increase the effect of gentamicin against *in*

vitro biofilms but also to reduce the risk of CRBSI, when associated with minocycline.[15,16] Using a recently developed rat model of totally implantable venous access ports (TIVAPs), we showed that the combination of gentamicin and EDTA led to the quick and long-lasting eradication of biofilms formed *in vivo* by *S. aureus*, *Staphylococcus epidermidis, Escherichia coli* and *P. aeruginosa.*[12,17] We also recently demonstrated that increasing the pH of a gentamicin-based lock solution with the clinically compatible alkaline amino-acid L-arginine led to the eradication of biofilms formed by *S. aureus* and *E. coli.*[13] Indeed, alkaline pH increased the effect of aminoglycosides against planktonic as well as biofilm persister cells, both *in vitro* and *in vivo.*[13] While these results were obtained with laboratory strains, we wondered whether these approaches could also be effective against a wide range of clinical strains responsible for CRBSI. Using clinical strains collected during a previously published prospective study, our main objective was to test *in vitro* the spectrum of action of the combination of gentamicin and EDTA or L-arginine.[18] We also studied other combinations including antibiotics that are commonly used in case of CRBSI caused by gentamicin-resistant strains, such as vancomycin or amikacin.[4,19,20]

Materials and methods

Bacterial strains and growth conditions. Between February 2009 and October 2010, we conducted a prospective study in Beaujon Hospital, a tertiary teaching hospital, during which 72 patients were included with a diagnosis of TIVAP-related infection.[18] Bacterial strains were collected and stored at -80°C. For the present study, we decided to focus on patients included with a diagnosis of TIVAP-related BSI, *i.e.* the most relevant clinical indication for ALT (**Supplementary Figure 1**).[4,8] We identified 43 cases of TIVAP-related BSI diagnosed at Beaujon and restricted our study to the most frequent bacterial pathogens responsible for CRBSI: Enterobacteriaceae*,* coagulase-negative staphylococci, *S. aureus*, *P. aeruginosa* and *Enterococcus faecalis*.[18] Among our strains, some did not resume growth when bacterial stocks were streaked on blood agar plates. As a result, we recovered 32 strains that have been further studied (**Table 1**).

Gram-positive bacteria (*S. aureus*, coagulase-negative staphylococci, *E. faecalis*) were grown in tryptic soy broth (TSB) supplemented with 0.25% (or 0.5% for *E. faecalis*) glucose (TSB glucose). Gram-negative bacteria (*P. aeruginosa* and Enterobacteriaceae*)* were grown in Lysogeny Broth (LB).[21] Unless specified, all chemicals and antibiotics were purchased from Sigma-Aldrich (St. Louis, MO). EDTA was prepared as follow. Briefly, 0.5M stock solution of disodium EDTA was prepared in water. Then, NaOH was added dropwise in order to reach a pH of ~8. EDTA was used at the final concentration of 30 mg/mL.

Determination of minimal inhibitory concentration Minimal inhibitory concentrations (MIC) were determined by broth microdilution in cation-adjusted Mueller-Hinton broth, as recommended by the Clinical and Laboratory Standards Institute (CLSI).[22,23] Stationary phase cultures were diluted 1:100 in fresh media and cultured at 37°C with agitation until reaching exponential growth. Then, bacterial inoculum was standardized up to a final concentration of

5.10^5 cfu/mL and exposed to serially diluted concentrations of antibiotics. Gentamicin and vancomycin were tested for Gram-positive bacteria. Gentamicin and amikacin were tested for Gram-negative bacteria. MIC was defined as the first well with no visible bacterial growth. The final value was the mean of 3 independent experiments. We used CLSI thresholds to define if a strain was susceptible or resistant towards one of the tested antibiotics (**Table 1**).[23]

***In vitro* biofilm formation.** *In vitro* biofilms were grown in triplicate for 24 hours (*S. aureus, S. epidermidis, E. faecalis* and Enterobacteriaceae) or 48 hours (*P. aeruginosa*) on UV-sterilized polyvinyl chloride (PVC) 96-well plates (Thermo Scientific, Rochester, NY), as previously described.[13,24] Briefly, stationary phase cultures were diluted up to OD_{600nm} of 0.05 in fresh media and 100µL of this inoculum was used in each well. Gram-positive bacterial biofilms were grown in TSB supplemented with 0.25% (or 0.5% for *E. faecalis*) glucose. Gram-negative bacterial biofilms were grown in LB broth. After 24 hours (or 48 hours for *P. aeruginosa*), planktonic bacteria were removed by 1X PBS washing and biofilms treated for 24 hours using different lock solutions (see below). After 24 hours, each well was washed twice with 1X PBS to remove planktonic bacteria and excess antibiotics and surviving cfu were quantified with a vigorous step of pipetting up and down in order to detach all biofilm bacteria from the surface of the wells. cfu were compared to 24h biofilms and expressed as % of survival.[13,24] For *S. aureus*, coagulase-negative staphylococci and Enterobacteriaceae, we decided to retain only the 4 most tolerant strains (*i.e.* the strains with the highest percentage of bacterial survival) after a 24-h exposure to the bactericidal antibiotic gentamicin at 5 mg/mL, a concentration that is usually recommended as ALT (**Supplementary Figure 2**).[8] Then, biofilms formed by the selected strains were treated using the same procedure with the following combinations: fresh media (control), gentamicin alone (5 mg/mL), EDTA alone (30 mg/mL), L-arginine alone (0.4%), gentamicin (5 mg/mL) + EDTA (30 mg/mL)

(GEN+EDTA), gentamicin (5 mg/mL) + L-arginine (0.4%) (GEN+L-arg). We also tested amikacin (5 mg/mL, for Gram-negative bacteria) or vancomycin (5 mg/mL, for Gram-positive bacteria) alone or associated with EDTA (30 mg/mL).

Statistical analysis. Each experiment was performed at least 3 times. Wilcoxon Mann-Whitney test (included in Graphpad Prism Version 5.04) was used to compare mortality of biofilm bacteria between each type of treatment. Different treatment groups were considered statistically different if p values were lower than 0.05. The combination of an antibiotic and an adjuvant was considered active if biofilm survival was significantly reduced, when compared with antibiotic treatment alone.

Results

EDTA-gentamicin lock is active against most tested clinical strains.

We first compared the activity of gentamicin alone or GEN+EDTA locks against *in vitro* biofilm formed in microtiter plate assay by clinical strains responsible for CRBSI. We observed that all tested strains exhibited various degrees of tolerance towards antibiotics with 0.01 to 50% of bacterial survival after a 24-h exposure to 5 mg/mL of gentamicin. Among the 18 tested clinical strains, all but one *P. aeruginosa* strain exhibited a significant reduction of bacterial survival when disodium EDTA was added to gentamicin (**Figure 1 to 5**). The effect was also seen against highly tolerant strains, *i.e.* strains with high % of survival when exposed to high concentration of gentamicin alone. For instance, even if 50% of *S. epidermidis* strain 50 biofilm bacteria survived after gentamicin challenge, the adjunction of EDTA increased bacterial mortality by 3-log (**Figure 2A**). Similar findings were made with highly tolerant strains of *S. aureus* (**Figure 1A**), *E. faecalis* (**Figure 3A**), *P. aeruginosa* (**Figure 4D**) and Enterobacteriaceae (**Figure 5A and D**). Lastly, the effect could also be seen against resistant strains, such as *P. aeruginosa* strain 32.

Taken together, these results demonstrate that the adjunction of disodium EDTA significantly increases the effect of gentamicin against biofilms formed by almost all tested strains of bacterial pathogens responsible for CRBSI, including highly tolerant or resistant bacteria.

The combination of L-arginine and gentamicin is active against all tested pathogens but *S. epidermidis*.

Against *S. aureus*, *E. faecalis,* Enterobacteriaceae and *P. aeruginosa*, GEN+L-arg lock is active against almost all tested strains, with the exception of one strain of *K. pneumoniae* (**Figure 1, 3, 4 and 5**). Conversely, the adjunction of L-arginine to gentamicin did not increase the effect of antibiotic alone against *S. epidermidis* (**Figure 2**). Against *S. aureus* or

P. aeruginosa, no significant difference could be seen regarding the reduction of biofilm survival comparing GEN+EDTA and GEN+L-arg locks (**Figure 1 and 4**).

Taken together, these results demonstrate that even if GEN+L-arg lock is active against *S. aureus*, *E. faecalis,* Enterobacteriaceae and *P. aeruginosa,* it does not significantly increase the effect of gentamicin against coagulase-negative staphylococci.

Use of EDTA also increases the efficiency of alternative antibiotic locks.

We then tested the effect of vancomycin or amikacin alone or associated with EDTA against our array of clinical strains to compare their activity on antibiotic resistant or susceptible bacteria.[19,20] We focused our study on EDTA, as it exhibited the wider spectrum of action. Against *S. aureus* or *E. faecalis*, gentamicin was associated with higher mortality of biofilm bacteria, as compared with vancomycin in all tested strains but 1 *S. aureus* (**Figure 1 and 3**). Conversely, against *S. epidermidis*, vancomycin was more active than gentamicin in 2 strains, less active in 1 strain and equally active in 1 strain (**Figure 2**). Against Gram-positive bacteria, the adjunction of EDTA increased mortality of biofilm bacteria, in all cases (**Figure 1 to 3**). However, in the case of *S. aureus*, the effect was related only to the effect of EDTA alone, as demonstrated by the absence of a significant difference between EDTA and VAN+EDTA (**Figure 1**). Conversely, against coagulase-negative staphylococci and *E. faecalis*, VAN+EDTA was more active than EDTA alone (**Figure 2 and 3**).

Against Gram-negative bacteria, amikacin was as active as gentamicin in 5/8 strains and more active than gentamicin in 3/8 strains (**Figure 4 and 5**). The adjunction of EDTA to amikacin increased the mortality of biofilm bacteria in 50% of cases (2 strains of *P. aeruginosa* and 2 strains of Enterobacteriaceae).

Taken together, these results demonstrated that the adjunction of EDTA to vancomycin or amikacin increased mortality of biofilm bacteria in a majority of clinical strains.

Discussion

The recent identification of gentamicin-based catheter locks (associated with EDTA or L-arginine) leading to fast and long-lasting eradication of biofilms formed by Gram-positive and Gram-negative pathogens suggests that these locks could be successfully used in clinical situations.[12,13] However, these adjuvant strategies were only tested on a limited number of laboratory bacterial strains and testing the efficiency of these locks against a wide and clinically relevant panel of strains responsible for CRBSI constitutes a mandatory preliminary towards potential clinical study. Here, we tested these 2 adjuvant strategies combining EDTA or L-arginine with aminoglycosides against 18 strains collected during a prospective study, specifically designed to study the clinical outcome after CRBSI.[18] We demonstrated that the adjuvant gentamicin + EDTA strategy was effective on a broader spectrum of Gram-positive and Gram-negative bacterial pathogens as compared to the adjuvant gentamicin + L-arginine strategy. Additionally, we showed that efficiency of other aminoglycosides such as amikacin (in Gram-negative bacteria) and vancomycin (in Gram-positive bacteria) are also potentiated by EDTA adjunction.

More specifically, we observed that the adjunction of EDTA significantly increases the effect of gentamicin against all tested strains but one *P. aeruginosa*. However, in this later strain, we also observed a trend toward a higher activity when GEN+EDTA was compared to gentamicin alone ($p=0.073$). We previously demonstrated that GEN+EDTA used as ALT was amenable to clinical studies as it eradicated biofilms formed by bacterial nosocomial pathogens.[12] Furthermore, another group also reported that GEN+EDTA was a promising combination for biofilm eradication.[15] The potentiation of gentamicin effect is very likely due to the ability of cation chelator to destabilize the biofilm matrix or because of a direct bactericidal effect of EDTA against biofilm bacteria.[14,25] In the present study, the fact that we do not reach biofilm eradication during *in vitro* experiments is very likely due to the short

course of lock treatment (24 hours), as compared to *in vivo* experiments (at least 5 days), as well as the presence of the immune system *in vivo* that may favor clearance of biofilm bacteria when weakened by the treatment. To date, no *in vitro*, *in vivo* or clinical data support the use of ALT during only one day. So far, a possible limitation for the use of EDTA is its commercial availability that is restricted to its association with minocycline.

We also tested another strategy using L-arginine as an adjuvant to gentamicin in order to increase bacterial persisters' mortality within biofilms.[13] Whereas L-arginine efficiently increased gentamicin activity against most tested bacteria, we did not observe any gentamicin potentiation against *S. epidermidis*. As *S. epidermidis* is a frequent pathogen in case of CRBSI, this limitation is important and should be taken into account before considering any clinical studies. One possible explanation regarding this observation is the frequent carriage of ACME (arginine catabolic mobile element) by coagulase-negative staphylococci. ACME frequently includes *arc,* a gene cluster encoding a complete additional arginine deiminase pathway.[26,27] ACME is found in more than 65% of methicillin-susceptible or resistant *S. epidermidis* strains.[26,28] Hence, one can hypothesize that, in *S. epidermidis*, increased arginine metabolism could reduce its adjuvant effect. Indeed, ACME is less frequently found in *S. aureus*, as compared with coagulase-negative staphylococci.[26,29]

We also observed an important variability between different strains within a single species regarding the effects of antibiotics alone or the magnitude of the synergistic effect. This observation highlights the importance of testing any candidate compound or combination against multiple strains representative of each bacterial species to rule out any strain-specific effect.

Three percents of methicillin-susceptible *S. aureus* and 11% of methicillin-resistant *S. aureus* were found to be gentamicin-resistant in a recent survey of hospital-acquired infections in Texas.[19] In Canadian intensive care units, 8% of *E. coli* and 32% of *P.*

aeruginosa were gentamicin-resistant.[20] More strikingly, 60% of *S. epidermidis* responsible for bloodstream infections in Germany were gentamicin-resistant.[30] Thus, identifying the most active approach against gentamicin-resistant bacteria is essential. *In vivo*, we previously showed that a 5-day GEN+EDTA ALT procedure allows the eradication of biofilm formed by gentamicin-resistant *S. aureus.*[12] Here, we also noticed that a synergistic effect could be seen *in vitro* between gentamicin and EDTA against gentamicin-resistant or intermediate strains, as shown with *P. aeruginosa* strain 32 and 35 or *S. epidermidis* strain 50. However, even with these strains, GEN+EDTA was still the most active combination. These data suggest that GEN+EDTA ALT could be used in case of gentamicin-resistant strain, even if more experimental data are required to confirm what is the best therapeutic strategy in this situation. Additionally, we wanted to study the efficiency of other clinically relevant combinations, such as AMK+EDTA against Gram-negative bacteria or VAN+EDTA against Gram-positive bacteria. Against Gram-positive bacteria, we noticed that the adjunction of EDTA increased biofilm mortality, in all cases. An effect was also seen against *S. epidermidis* strain 53, despite a high vancomycin MIC, confirming that planktonic bacteria-based antibiotic susceptibility tests do not predict biofilm tolerance towards antibiotics. Against Gram-negative bacteria, a synergy between EDTA and amikacin was noticed in 50% of cases and no antagonism was seen. Such an effect was also observed even in the case of an amikacin-intermediate strain, such as *P. aeruginosa* strain 32. Such locks could potentially be used in case of resistance towards gentamicin.

Few studies compared the activity of gentamicin to other drugs against biofilms using a standardized method. Against *S. epidermidis* and *S. aureus*, it has been shown that vancomycin was more active that gentamicin in biofilm setting.[31,32] However, *in vivo*, gentamicin at 40 mg/mL was shown to be more active that vancomycin at 2 mg/mL against *S. aureus.*[33] In the present study, gentamicin was more active than vancomycin against *S. aureus*

or *E. faecalis* biofilms. The results were less clear-cut in the case of *S. epidermidis*, since vancomycin was more active than gentamicin in 2 strains, less active in 1 strain and equally active in 1 strain. Against Gram-negative bacteria, amikacin was as active as gentamicin in 5 out of 8 strains but more active than gentamicin in the remaining 3 strains. However, no other *in vitro* or *in vivo* study compared the activity of gentamicin to amikacin as locks against Gram-negative bacteria.

To note, the characteristics of the surface that is used for biofilm formation might influence the phenotype of tolerance towards antibiotics. In our case, the surface of the 96-well PVC plates differs from that of a silicone catheter and might be a limitation of the present study. Other technical limitations are the use of vigorous pipetting up and down for cfu quantification and the assessment of bacterial mortality at a single time point.

In conclusion, our data demonstrate that EDTA acts synergistically with gentamicin to kill biofilms formed by bacterial strains responsible for CRBSI. A clinical study assessing the potential of GEN+EDTA as a lock therapy is now warranted.

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Transparency declarations

Conflict of interest: none to declare

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