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David Lebeaux, Véronique Leflon-Guibout, Jean-Marc Ghigo, Christophe Beloin. In vitro activity of gentamicin, vancomycin or amikacin combined with EDTA or l-arginine as lock therapy against a wide spectrum of biofilm-forming clinical strains isolated from catheter-related infections.. *Journal of Antimicrobial Chemotherapy*, 2015, 70 (6), pp.1704-1712. 10.1093/jac/dkv044 . pasteur-01378735

HAL Id: pasteur-01378735

<https://pasteur.hal.science/pasteur-01378735v1>

Submitted on 21 Nov 2016

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

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5

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16

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

18

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

21

22 **Abstract**

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

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

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

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

48

49 **Introduction**

50 Following an initial report in 1988¹, several studies demonstrated that antibiotic lock therapy
51 (ALT) could be a therapeutic option in case of catheter-related bloodstream infection
52 (CRBSI).¹⁻³ ALT relies on the instillation of a small volume of highly concentrated antibiotic
53 solution that dwells in the lumen of the catheter for 12 to 72 hours, in order to eradicate
54 biofilm formed on the inner surface of the device.^{1,4} Indeed, most of treatment difficulties
55 encountered during CRBSI are related to the presence of high cell density bacterial
56 communities called bacterial biofilms.⁵ Biofilms display characteristic properties, including
57 high tolerance towards antimicrobials that is defined by the ability of a subset of bacteria to
58 survive in the presence of high concentration of antibiotics.^{6,7}

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

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

74 *vitro* biofilms but also to reduce the risk of CRBSI, when associated with minocycline.^{15,16}
75 Using a recently developed rat model of totally implantable venous access ports (TIVAPs),
76 we showed that the combination of gentamicin and EDTA led to the quick and long-lasting
77 eradication of biofilms formed *in vivo* by *S. aureus*, *Staphylococcus epidermidis*, *Escherichia*
78 *coli* and *P. aeruginosa*.^{12,17} We also recently demonstrated that increasing the pH of a
79 gentamicin-based lock solution with the clinically compatible alkaline amino-acid L-arginine
80 led to the eradication of biofilms formed by *S. aureus* and *E. coli*.¹³ Indeed, alkaline pH
81 increased the effect of aminoglycosides against planktonic as well as biofilm persister cells,
82 both *in vitro* and *in vivo*.¹³ While these results were obtained with laboratory strains, we
83 wondered whether these approaches could also be effective against a wide range of clinical
84 strains responsible for CRBSI. Using clinical strains collected during a previously published
85 prospective study, our main objective was to test *in vitro* the spectrum of action of the
86 combination of gentamicin and EDTA or L-arginine.¹⁸ We also studied other combinations
87 including antibiotics that are commonly used in case of CRBSI caused by gentamicin-
88 resistant strains, such as vancomycin or amikacin.^{4,19,20}
89

90 **Materials and methods**

91 **Bacterial strains and growth conditions.** Between February 2009 and October 2010, we
92 conducted a prospective study in Beaujon Hospital, a tertiary teaching hospital, during which
93 72 patients were included with a diagnosis of TIVAP-related infection.¹⁸ Bacterial strains
94 were collected and stored at -80°C. For the present study, we decided to focus on patients
95 included with a diagnosis of TIVAP-related BSI, *i.e.* the most relevant clinical indication for
96 ALT (**Supplementary Figure 1**).^{4,8} We identified 43 cases of TIVAP-related BSI diagnosed
97 at Beaujon and restricted our study to the most frequent bacterial pathogens responsible for
98 CRBSI: Enterobacteriaceae, coagulase-negative staphylococci, *S. aureus*, *P. aeruginosa* and
99 *Enterococcus faecalis*.¹⁸ Among our strains, some did not resume growth when bacterial
100 stocks were streaked on blood agar plates. As a result, we recovered 32 strains that have been
101 further studied (**Table 1**).

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

109

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

115 5.10⁵ cfu/mL and exposed to serially diluted concentrations of antibiotics. Gentamicin and
116 vancomycin were tested for Gram-positive bacteria. Gentamicin and amikacin were tested for
117 Gram-negative bacteria. MIC was defined as the first well with no visible bacterial growth.
118 The final value was the mean of 3 independent experiments. We used CLSI thresholds to
119 define if a strain was susceptible or resistant towards one of the tested antibiotics (**Table 1**).²³

120

121 ***In vitro* biofilm formation.** *In vitro* biofilms were grown in triplicate for 24 hours (*S. aureus*,
122 *S. epidermidis*, *E. faecalis* and Enterobacteriaceae) or 48 hours (*P. aeruginosa*) on UV-
123 sterilized polyvinyl chloride (PVC) 96-well plates (Thermo Scientific, Rochester, NY), as
124 previously described.^{13,24} Briefly, stationary phase cultures were diluted up to OD_{600nm} of 0.05
125 in fresh media and 100µL of this inoculum was used in each well. Gram-positive bacterial
126 biofilms were grown in TSB supplemented with 0.25% (or 0.5% for *E. faecalis*) glucose.
127 Gram-negative bacterial biofilms were grown in LB broth. After 24 hours (or 48 hours for *P.*
128 *aeruginosa*), planktonic bacteria were removed by 1X PBS washing and biofilms treated for
129 24 hours using different lock solutions (see below). After 24 hours, each well was washed
130 twice with 1X PBS to remove planktonic bacteria and excess antibiotics and surviving cfu
131 were quantified with a vigorous step of pipetting up and down in order to detach all biofilm
132 bacteria from the surface of the wells. cfu were compared to 24h biofilms and expressed as %
133 of survival.^{13,24} For *S. aureus*, coagulase-negative staphylococci and Enterobacteriaceae, we
134 decided to retain only the 4 most tolerant strains (*i.e.* the strains with the highest percentage of
135 bacterial survival) after a 24-h exposure to the bactericidal antibiotic gentamicin at 5 mg/mL,
136 a concentration that is usually recommended as ALT (**Supplementary Figure 2**).⁸ Then,
137 biofilms formed by the selected strains were treated using the same procedure with the
138 following combinations: fresh media (control), gentamicin alone (5 mg/mL), EDTA alone (30
139 mg/mL), L-arginine alone (0.4%), gentamicin (5 mg/mL) + EDTA (30 mg/mL)

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

143

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

150

151 **Results**

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

153 We first compared the activity of gentamicin alone or GEN+EDTA locks against *in vitro*
154 biofilm formed in microtiter plate assay by clinical strains responsible for CRBSI. We
155 observed that all tested strains exhibited various degrees of tolerance towards antibiotics with
156 0.01 to 50% of bacterial survival after a 24-h exposure to 5 mg/mL of gentamicin. Among the
157 18 tested clinical strains, all but one *P. aeruginosa* strain exhibited a significant reduction of
158 bacterial survival when disodium EDTA was added to gentamicin (**Figure 1 to 5**). The effect
159 was also seen against highly tolerant strains, *i.e.* strains with high % of survival when exposed
160 to high concentration of gentamicin alone. For instance, even if 50% of *S. epidermidis* strain
161 50 biofilm bacteria survived after gentamicin challenge, the adjunction of EDTA increased
162 bacterial mortality by 3-log (**Figure 2A**). Similar findings were made with highly tolerant
163 strains of *S. aureus* (**Figure 1A**), *E. faecalis* (**Figure 3A**), *P. aeruginosa* (**Figure 4D**) and
164 Enterobacteriaceae (**Figure 5A and D**). Lastly, the effect could also be seen against resistant
165 strains, such as *P. aeruginosa* strain 32.

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

169

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

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

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

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

181

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

183 We then tested the effect of vancomycin or amikacin alone or associated with EDTA against
184 our array of clinical strains to compare their activity on antibiotic resistant or susceptible
185 bacteria.^{19,20} We focused our study on EDTA, as it exhibited the wider spectrum of action.

186 Against *S. aureus* or *E. faecalis*, gentamicin was associated with higher mortality of biofilm
187 bacteria, as compared with vancomycin in all tested strains but 1 *S. aureus* (**Figure 1 and 3**).

188 Conversely, against *S. epidermidis*, vancomycin was more active than gentamicin in 2 strains,
189 less active in 1 strain and equally active in 1 strain (**Figure 2**). Against Gram-positive

190 bacteria, the adjunction of EDTA increased mortality of biofilm bacteria, in all cases (**Figure**
191 **1 to 3**). However, in the case of *S. aureus*, the effect was related only to the effect of EDTA

192 alone, as demonstrated by the absence of a significant difference between EDTA and
193 VAN+EDTA (**Figure 1**). Conversely, against coagulase-negative staphylococci and *E.*

194 *faecalis*, VAN+EDTA was more active than EDTA alone (**Figure 2 and 3**).

195 Against Gram-negative bacteria, amikacin was as active as gentamicin in 5/8 strains and more
196 active than gentamicin in 3/8 strains (**Figure 4 and 5**). The adjunction of EDTA to amikacin

197 increased the mortality of biofilm bacteria in 50% of cases (2 strains of *P. aeruginosa* and 2
198 strains of Enterobacteriaceae).

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

201

202 **Discussion**

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

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

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

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

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

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

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

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

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

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

288

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

292

293

294 **Acknowledgements**

295 None.

296

297 **Fundings**

298 This work was supported by Institut Pasteur grant and by the French Government's
299 Investissement d'Avenir program, Laboratoire d'Excellence "Integrative Biology of Emerging
300 Infectious Diseases" (grant n°ANR-10-LABX-62-IBEID) and from Fondation pour la
301 Recherche Médicale grant "Equipe FRM DEQ20140329508". D.L. was supported by a grant
302 from the AXA Research Fund.

303

304

305 **Transparency declarations**

306 Conflict of interest: none to declare

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