



HAL
open science

Full and broad-spectrum in vivo eradication of catheter-associated biofilms using gentamicin-EDTA antibiotic lock therapy.

Ashwini Chauhan, David Lebeaux, Jean-Marc Ghigo, Christophe Beloin

► **To cite this version:**

Ashwini Chauhan, David Lebeaux, Jean-Marc Ghigo, Christophe Beloin. Full and broad-spectrum in vivo eradication of catheter-associated biofilms using gentamicin-EDTA antibiotic lock therapy.. *Antimicrobial Agents and Chemotherapy*, 2012, 56 (12), pp.6310-8. 10.1128/AAC.01606-12 . pasteur-01126606

HAL Id: pasteur-01126606

<https://pasteur.hal.science/pasteur-01126606v1>

Submitted on 6 Mar 2015

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial - NoDerivatives 4.0 International License

Full and broad-spectrum *in vivo* eradication of catheter-associated biofilms using gentamicin-EDTA antibiotic lock therapy

1

2 Ashwini Chauhan, David Lebeaux, Jean-Marc Ghigo and Christophe Beloin[#]

3

4 *Institut Pasteur, Unité de Génétique des Biofilms, Département de Microbiologie F-75015 Paris,*
5 *France*

6

7 **# Corresponding author**

8 **E-mail:** cbeloin@pasteur.fr

9 **Tel:** (+33) 01 44 38 95 97

10 **Fax** (+33) 01 45 68 88 36

11

12 **Full-length article**

13 **Abstract:** 245 words

14 **Figure:** 5

15 **Table:** 1

16 **References:** 54

17

18 **Running title:** Eradication of *in vivo* TIVAP-associated biofilms

19 **Keywords:** Biofilm; eradication; catheter-related infection; chelator; antibiotic lock therapy

20 **Abbreviations:** ALT: antibiotic lock therapy; CFU: colony-forming unit; CRBSI: catheter-related
21 bloodstream infection; EDTA: ethylenediamine-tetra-acetic acid; EM: electron microscopy; TIVAP:
22 totally implantable venous access port.

23

24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47

ABSTRACT

Biofilms that develop on indwelling devices are a major concern in clinical settings. While removal of colonized devices remains the most frequent strategy for avoiding device-related complications, antibiotic lock therapy constitutes an adjunct therapy for catheter-related infection. However, currently used antibiotic lock solutions are not fully effective against biofilms, thus warranting a search for new antibiotic locks. Metal binding chelators have emerged as potential adjuvants due to their dual anticoagulant/antibiofilm activities but studies investigating their efficiency were mainly *in vitro* or else focused on their effects in prevention of infection. To assess the ability of such chelators to eradicate mature biofilms, we used an *in vivo* model of totally implantable venous access port inserted in rats and colonized either by *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli* or *Pseudomonas aeruginosa*. We demonstrate that use of tetrasodium EDTA (30 mg/mL) as a supplement to the gentamicin (5 mg/mL) antibiotic lock solution associated with systemic antibiotics completely eradicated Gram-positive and Gram-negative bacterial biofilms developed in totally implantable venous access ports. Gentamicin-EDTA lock was able to eliminate biofilms with a single instillation, thus reducing length of treatment. Moreover, we show that this combination was effective for immuno-suppressed rats. Lastly, we demonstrate that gentamicin-EDTA lock is able to eradicate the biofilm formed by a gentamicin-resistant strain of methicillin-resistant *S. aureus*. This *in vivo* study demonstrates the potential of EDTA as an efficient antibiotic adjuvant to eradicate catheter-associated biofilms of major bacterial pathogens and thus provides a promising new lock solution.

48 INTRODUCTION

49 Central venous catheters are routinely used to administer medication or fluids to patients admitted to
50 oncology, nephrology and intensive care units (2, 46, 51). Although these devices greatly improve
51 patient health, their use is often associated with medical complications due to colonization by
52 pathogenic microorganisms (37). This leads to development of complex bacterial or fungal biofilm
53 communities that display strong tolerance towards antimicrobials (14, 36, 44). Biofilms are difficult to
54 eradicate; moreover, they constitute a potential source of bloodstream infections, a leading cause of
55 healthcare-associated infections in critically ill patients (28). Currently, there is no fully efficient method
56 for treating catheter-related biofilms aside from traumatic and costly removal of colonized devices (5,
57 10, 41, 49). However, recent clinical practice guidelines recommended the use of antibiotic lock
58 therapy (ALT) for treatment of uncomplicated long-term catheter-related infections (30). ALT relies on
59 the instillation of highly concentrated antibiotic solutions (up to 1,000 times the minimal inhibitory
60 concentration (MIC)), left to dwell in the catheter for 12 to 24 h in order to prevent or eradicate biofilm
61 formation. Although ALT shows a high success rate for coagulase-negative staphylococci and Gram-
62 negative bacterial catheter-related infections (13, 17, 20), catheter removal is still recommended for
63 pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* due to lack of efficient
64 antibiotic lock or frequent hematogenous complications (19, 30).

65 Growing concern over drug-resistant pathogens (31), combined with increasing use of central venous
66 catheters, has led to evaluation of novel lock solutions. While some studies showed that combinations
67 of different antibiotics are more efficient than single-antibiotic lock solutions (1, 9, 33), the efficacy of
68 non-antibiotic compounds is currently being investigated to improve lock solutions and reduce use of
69 antibiotics.

70 The anticoagulant heparin is the most widely used ALT adjuvant for reducing catheter colonization and
71 related infection (7, 29). However, heparin activity is also reported to be impaired in gentamicin
72 solutions (15, 47). Other compounds having both anticoagulant and chelating properties, such as
73 sodium citrate and ethylenediamine-tetraacetic acid (EDTA) have been proposed as anti-biofilm ALT
74 adjuvants (35, 43, 50). For instance, association of EDTA and gentamicin demonstrated a potent
75 activity against *in vitro* biofilms formed by *S. aureus*, *Staphylococcus epidermidis* and *P. aeruginosa*
76 (6). In addition, preventive EDTA-minocycline locks were shown to reduce the incidence of long term
77 catheter-related infections in clinical studies (4, 6, 34, 38).

78 However, despite encouraging results in preventive approaches, only limited *in vivo* data have
79 validated the use of these non-antibiotic ALT adjuvants. In this study, we evaluated the curative
80 efficacy of a tetrasodium EDTA-gentamicin ALT solution against bacterial biofilms formed in an *in vivo*
81 model of totally implantable venous access ports (TIVAP) (9). While gentamicin, EDTA alone and a
82 70% ethanol lock solution were not completely effective against TIVAP-associated biofilms, we
83 showed that single-dose treatment with a gentamicin-EDTA lock solution fully eradicated both Gram-
84 positive and Gram-negative bacterial catheter biofilms. Novel anti-biofilm strategies are urgently
85 needed to improve treatment of catheter-related infections and patient outcome with reduced length of
86 antibiotic exposure. Gentamicin-EDTA may lead to salvaging colonized catheters over a short duration,
87 thus directly impacting length of hospital stay, morbidity and health-care costs.

88

89

90

91

92 **MATERIALS AND METHODS**
93

94 **Bacterial strains.** Luminescent variants of four clinically relevant pathogens, i.e. *S. aureus*, *S.*
95 *epidermidis*, *P. aeruginosa* and *Escherichia coli*, were either purchased (*S. aureus* MSSA Xen36,
96 MRSA Xen31 and *S. epidermidis* Xen43 from Caliper) or donated (*P. aeruginosa* Lm1, a
97 bioluminescent derivative of the PAK clinical strain (39)) and *E. coli* EAEC 55989 transformed with
98 stable plasmid pAT881 (18). *S. epidermidis* Xen43 is derived from *S. epidermidis* 1457 (52), a
99 methicillin-susceptible strain (27). *S. aureus* Xen36 and Xen31 and *S. epidermidis* Xen43 were
100 cultured in tryptic soy broth (TSB) supplemented with 0.25% glucose, while *E. coli* and *P. aeruginosa*
101 strains were grown in lysogeny broth (LB) at 37°C.

102
103 **Determination of in vitro minimal inhibitory concentrations (MICs).** MICs were determined by
104 broth microdilution as per CLSI (Methods for dilution of antimicrobial susceptibility tests for bacteria
105 that grow aerobically; Approved Standard, Eighth Edition. CLSI Document M07-A8. Wayne, PA:
106 Clinical and Laboratory Standards Institute; 2009). Briefly, exponentially growing bacteria were diluted
107 to a final inoculum of 5×10^5 bacteria/mL. Results were read after 16 to 18 h of culture. MIC was
108 defined as the first well without visible growth. MIC was performed in TSB glucose at 0.25% for *S.*
109 *aureus* and *S. epidermidis* and in LB for *E. coli* and *P. aeruginosa* (Table 1).

110
111 **Antibacterial agents.** Gentamicin sulfate, vancomycin hydrochloride, EDTA-tetrasodium salt and
112 ethanol (70%) were purchased from Sigma-Aldrich, Inc. Distilled water was purchased from Gibco for
113 preparing antibacterial solutions.

114
115 **Animal model.** Male CD/SD (IGS:CrI) rats purchased from Charles River weighed 275-300 g and
116 were allowed to acclimatize using 12 h day/night cycles for one week before use at the Institut Pasteur
117 animal facilities accredited by the French Ministry of Agriculture to perform experiments on live rodents
118 (accreditation #A75-15 27, issued on November 12, 2004 and #A75-15 04, issued on May 22, 2008),
119 in compliance with French and European regulations on care and protection of laboratory animals (EC
120 Directive 86/609, French Law 2001-486 issued on June 6, 2001). Protocols were approved by the
121 veterinary staff of the Institut Pasteur animal facility and were performed in compliance with NIH
122 Animal Welfare Insurance #A5476-01 issued on July 2, 2007.

123 *Catheter placement.* TIVAP implantation was performed as described previously (9). Briefly, surgical
124 placement of TIVAP in anesthetized rats was carried out as follows: the port was implanted at the
125 dorsal midline toward the lower end of the thoracic vertebrae by creating a subcutaneous pocket. The
126 catheter was tunneled subcutaneously into the ventral side in the clavicle region, inserted into the
127 jugular vein by a micro-incision and progressively inserted into the superior vena cava up to the right
128 atrium. Patency of TIVAP was maintained by flushing 1X sterile PBS followed by heparin lock (500
129 IU/mL) every day. Prior to inoculation of clinical strains, all rats were checked for the absence of
130 infection by plating 100 μ L blood, as well as monitoring for the absence of any luminescence signals.

131

132 ***Inoculation of TIVAP in immunocompetent rats.*** A previously optimized (9) inoculum dose of 10^6
133 cells of *S. aureus* MSSA Xen36, 10^8 cells of *S. aureus* MRSA Xen31 or *S. epidermidis* Xen43, 10^4
134 cells of *E. coli* pAT881 and 10^6 cells of *P. aeruginosa* in 100 μ L 1X PBS, was injected through a
135 silicone septum into the port using a Huber needle. Overnight cultures were diluted in 1X PBS to the
136 optimized inoculum dose. The inoculum size was also confirmed by plating it for CFU/mL on
137 respective antibiotic plates. Control rats received 1X PBS. Colonization of the TIVAP was monitored
138 using the IVIS-100 imaging system (Xenogen Corporation, Alameda, CA, USA).

139

140 ***Immune suppression and infection in catheterized rats.*** The immune system of the rats was
141 suppressed using cyclophosphamide (Sigma Aldrich cat# C0768-5G). The optimized dose and
142 regimen of cyclophosphamide delivery, determined by estimating total blood leukocyte count using the
143 animal blood cell counter Vet ABC (SCIL, Germany), was used as described previously (9). The
144 hundred mg/kg body weight of cyclophosphamide was finally selected for giving intraperitoneal
145 injections to rats on day -4 of inoculation. The inoculum dose of 10^2 MSSA Xen36 cells/100 μ L 1X PBS
146 was used for TIVAP inoculation and confirmed by plating for CFU/mL. Control catheterized and
147 immunosuppressed rats received 100 μ L 1X PBS only. Prior to inoculation of clinical strains, all rats
148 were checked for the absence of infection, as for immunocompetent rats.

149

150 ***Extraction and quantification of viable bacteria from the biofilm.*** TIVAP were carefully wiped with
151 70% ethanol before extracting intraluminal biofilm bacteria to avoid contaminants. The catheter was
152 cut into small pieces and a slit was made horizontally to expose the lumen; it was next transferred to a

153 tube containing 0.5 mL sterile 1X phosphate-buffered saline (PBS). The septum was removed from
154 the port using a sterile scalpel and forceps, cut into small pieces and transferred to a separate tube
155 containing 0.5 mL sterile 1X PBS. Cells attached to the titanium body of the port were scratched in
156 100 μ L 1X PBS and transferred to the same tube as the septum. Biofilm that formed on the septum
157 and in the lumen of the catheter was extracted by vigorously vortexing the tubes for 1 min, followed by
158 transfer to an ultrasonic water bath (NEYtech Ultrasonik, 44-48 Khz) for 5 min. Bacterial suspensions
159 from the tubes were then mixed to analyze total CFU/mL/TIVAP. The bacterial suspension was then
160 serially diluted, plated on agar plates and incubated at 37°C for colony counts.

161

162 ***In vivo ALT.*** We had previously shown that treatment of TIVAP colonized by biofilm with ALT alone
163 can lead to systemic infection, therefore causing death of the animals (9). Thus, in this study, ALT was
164 always used in conjunction with systemic vancomycin (50 mg/kg, for MSSA, MRSA and *S.*
165 *epidermidis*) or gentamicin (30 mg/kg, for Gram-negative bacteria) subcutaneous injections (48, 54).
166 The efficacy of gentamicin (5 mg/mL), EDTA (30 mg/mL), ethanol (70%) and gentamicin-EDTA (5
167 mg/mL-30 mg/mL) lock therapy was evaluated. All lock solutions were prepared in sterile distilled
168 water. The 3-day-old biofilm formed inside the implanted TIVAP was locked with 200 μ L of the above-
169 mentioned antibiotics following 2 types of regimen. First, a 5-day ALT regimen during which the old
170 lock was replaced by a new one every 24 h for 5 days in conjunction with systemic treatment for 5
171 days. We also assessed a 1-day ALT regimen with a single instillation of ALT dwelling for 7 days in
172 conjunction with 1 day of systemic treatment. We monitored biofilm clearance by luminescence
173 imaging and rats were sacrificed after day 7 of the last ALT instillation for estimating viable cell counts
174 and electron microscopy analyses. Immunosuppressed rats were sacrificed on day 3 post-ALT
175 instillation. Rats with a colonized TIVAP but receiving PBS ALT were used as controls.

176

177 ***Statistical analysis.*** Results for CFU are mean +/- standard deviation. Statistical differences were
178 evaluated using one-way ANOVA (Tukey multiple comparison test) included in Graphpad Prism
179 Version 5.0c. The treatment groups were considered statistically different if p-values were lower than
180 0.05.

181

182 **RESULTS**

183

184 **Gentamicin-EDTA efficacy against *S. aureus* *in vivo* catheter-associated biofilms.**

185 To evaluate the *in vivo* efficacy of tetrasodium EDTA as a potential adjuvant in ALT, we tested
186 different gentamicin-based lock solutions against bioluminescent methicillin-susceptible *S. aureus*
187 (MSSA) biofilms growing in TIVAP implanted in rats (n=5 rats for each treatment). In addition to
188 solutions containing gentamicin alone, EDTA alone or combined gentamicin-EDTA, we evaluated 70%
189 ethanol, which was shown to be an effective antibacterial lock agent both *in vitro* and *in vivo* (11, 23).
190 We first used ALT instillations renewed every 24 h for 5 days in conjunction with systemic vancomycin
191 injections (see Materials and methods) and we monitored *in vivo* bacterial clearance as a function of
192 luminescence. While luminescence could still be detected in rats with PBS ALT, ethanol, gentamicin or
193 EDTA alone, no signal was captured from rats treated with the gentamicin-EDTA lock (Figure 1A-F).
194 These results were confirmed by the bacterial count. While a high load of MSSA (8.6-log CFU/mL)
195 was recovered from TIVAP of rats with PBS ALT (Figure 1G), gentamicin or EDTA ALT alone reduced
196 MSSA CFU recovered from TIVAP biofilms to 4.3 to 5.3-log CFU/mL and 4.2 to 6.1-log CFU/mL,
197 respectively (Figure 1G). Similarly, 70% ethanol ALT could only clear biofilm in one out of 5 rats; in the
198 remaining four rats, 4.2 to 5.9-logs CFU/mL were still recovered from TIVAP 7 days post-ALT
199 treatment (Figure 1G). Although gentamicin or EDTA alone could not completely eradicate MSSA
200 biofilm, the absence of viable cell counts from TIVAP 7 days post-gentamicin-EDTA ALT confirmed its
201 antibiofilm efficacy (Figure 1G).

202 While our results demonstrated the *in vivo* efficacy of a 5-day regimen of gentamicin-EDTA ALT
203 treatment against MSSA biofilms, we also sought to determine whether gentamicin-EDTA ALT would
204 reduce the length and frequency of ALT treatment. For this, we applied a single instillation of
205 gentamicin-EDTA lock solution in conjunction with systemic vancomycin injections on 3-day-old *in vivo*
206 MSSA biofilm (1-day regimen). After 1 day, we already observed an absence of luminescence and a
207 ~4.2-log reduction in bacterial CFU/mL compared to rats with PBS ALT (day 1 in Figure 2A, 2C and
208 2E). Moreover, analysis of TIVAP 7 days after single gentamicin-EDTA ALT left to dwell for 7 days
209 (day 8) showed complete removal of bacteria from the catheters (day 8 in Figure 2A, 2C, 2D, and
210 Figure 2E). Although a decrease in bioluminescence was observed, rats with PBS ALT displayed a 2-
211 log-increase in bacterial colonization (Figure 2B and 2E). Indeed, bioluminescence will not detect
212 bacteria that are viable but are either dormant or growing anaerobically in TIVAP (9). Furthermore, the

213 absence of bacteria in gentamicin-EDTA-treated TIVAP was confirmed by scanning electron
214 microscopy. While TIVAP extracted from rats treated with either gentamicin or EDTA alone showed
215 the presence of biofilms with a dense bacterial population (Figure 2F-G), TIVAP from rats treated with
216 gentamicin-EDTA ALT displayed only a meshwork of host-derived fibrin-like material and the absence
217 of bacteria (Figure 2H).

218 These results demonstrated that use of a single instillation of gentamicin-EDTA antibiotic lock solution
219 successfully eradicated catheter-associated biofilms formed *in vivo* by MSSA.

220

221 **One-shot gentamicin-EDTA ALT is effective in immunosuppressed animals.**

222 Immunosuppressed patients are highly susceptible to bloodstream infections associated with central
223 venous catheter colonization (45). In order to evaluate whether gentamicin-EDTA ALT might also be
224 useful in immunosuppressed hosts, TIVAP-implanted rats were treated with cyclophosphamide prior to
225 inoculating them with 10^2 CFU/100 μ L of MSSA in the TIVAP (number of rats, n=3). TIVAP-associated
226 biofilms that developed after 3 days were treated with a single instillation of PBS, gentamicin or
227 gentamicin-EDTA lock in conjunction with systemic treatment. We showed that rats with PBS ALT
228 displayed luminescent signals corresponding to ~ 7.9 -log CFU/mL and died by day 3 despite
229 concomitant systemic treatment with vancomycin (Figure 3A-B and G). Moreover, rats (n=3) that
230 received only gentamicin ALT survived, but continued to display ~ 5.1 -log CFU/mL in the lumen of
231 TIVAP collected 3 days after ALT (Figure 3C-D and G). In contrast, immunosuppressed rats (n=3)
232 treated with a single instillation of a gentamicin-EDTA lock in conjunction with systemic treatment
233 showed 100% survival, and complete eradication of MSSA biofilm from TIVAP was noted 3 days after
234 treatment (Figure 3E-F and G). These results therefore demonstrated the *in vivo* efficacy of the
235 gentamicin-EDTA lock solution both in immunocompetent and immunosuppressed animals.

236

237 **Efficacy of gentamicin-EDTA ALT against *S. epidermidis* and methicillin-resistant *S. aureus*.**

238 In addition to MSSA infections, staphylococci-associated biofilm infections may also be due to
239 methicillin-resistant (MRSA) strains, while *S. epidermidis* is the most commonly reported bacterium in
240 catheter-related infections (9, 24, 30). To test the efficacy of a single instillation of gentamicin-EDTA
241 against *S. epidermidis* and MRSA, we used two clinical bioluminescent strains of *S. epidermidis*
242 (Xen43) and MRSA (Xen31). These two strains are poorly luminescent *in vivo* and therefore did not

243 enable us to non-invasively monitor biofilm colonization in implanted TIVAP; however, both of them led
244 to formation of *in vivo* biofilm (Figure 4 and (9)). Although a single instillation of gentamicin-EDTA (1-
245 day ALT regimen) eradicated *S. epidermidis* biofilm, ~ 3 -log CFU/mL bacteria could still be recovered
246 from TIVAP-associated MRSA biofilms (number of animals, n=3) (Figure 4). However, using a 5-day
247 ALT regimen, we demonstrated that *in vivo* TIVAP-associated MRSA biofilms could be successfully
248 eradicated with gentamicin-EDTA ALT, compared with gentamicin-treated biofilms where ~ 6.2 -log
249 CFU/mL bacteria were recovered (number of animals, n= 3) (Figure 4).

250

251 **The gentamicin-EDTA lock solution is efficient against Gram-negative bacteria.**

252 Although a high treatment success rate has been recently shown in the case of Gram-negative
253 bacteria catheter-related bloodstream infections (20), current guidelines suggest removal of catheters
254 colonized by Gram-negative pathogens having a propensity for biofilm formation, such as *P.*
255 *aeruginosa* (30). To test the potential of a combined gentamicin-EDTA lock against frequent catheter-
256 associated Gram-negative pathogens such as *E. coli* and *P. aeruginosa*, we used a single instillation
257 of gentamicin-EDTA ALT 3 days after inoculation with *E. coli* or *P. aeruginosa*, alongside systemic
258 gentamicin treatment. We observed complete eradication of *E. coli* and *P. aeruginosa* TIVAP-
259 associated biofilms using the combined gentamicin-EDTA lock solution, as indicated by the absence
260 of luminescent signals within 24 h of treatment (Figure 5A-D and 5E-H). Moreover, no bacteria were
261 recovered from the catheters harvested after 7 days post-treatment, while control rats continued to
262 display ~ 9.5 -log CFU/mL for *E.coli* and ~ 8.7 -log CFU/mL for *P. aeruginosa* (Figure 5I).

263

264 Taken together, these results demonstrate the potential of a gentamicin-EDTA combination as
265 a broad-spectrum anti-biofilm lock solution, not only against Gram-positive but also against Gram-
266 negative catheter-associated biofilm-forming pathogens.

267

268 **DISCUSSION**

269
270 Significant progress has been made in clinical handling of central venous catheters, but the
271 development of pathogenic biofilms remains a major problem with severe clinical implications (30, 37).
272 In the case of intermittently used devices, ALT is a widely used strategy recommended for prevention
273 or cure of intraluminal catheter-associated biofilms (30, 40). Although current ALT have significant
274 effects on catheter handling, use of adjuvant molecules is under investigation at present to potentiate
275 existing antibiotic treatment against biofilms for preventing and curing catheter-related bloodstream
276 infections (CRBSI) (30, 40).

277 Recent *in vitro* studies demonstrated that metal chelators such as EDTA and citrate, which bind to
278 metal cations such as Ca^{2+} , Fe^{3+} and Mg^{2+} , act both as anticoagulant and antibiofilm agents and
279 therefore enhance the antimicrobial effect of antibiotics (3, 6, 35, 53).

280 In the present study, using our previously optimized rat model with an implanted TIVAP, we evaluated
281 the *in vivo* efficacy of the anticoagulant chelator tetrasodium EDTA, in combination with gentamicin, as
282 a potential curative antibiotic lock solution (9). For initial evaluation of EDTA as an adjuvant to the
283 gentamicin lock solution as compared to gentamicin alone, EDTA alone or ethanol, we chose *S.*
284 *aureus* catheter colonization, for which catheter removal is mandatory (16, 19). We showed that the
285 gentamicin-EDTA combination was the most effective lock solution compared to gentamicin alone,
286 EDTA alone or ethanol (70%). Since, we previously demonstrated the risk of systemic infection when
287 ALT was used alone, we always used systemic antibiotics alongside (9). We chose systemic
288 vancomycin, even in case of methicillin-susceptible *Staphylococcus* spp. in order to compare the
289 effect of ALT between these different strains without having a bias related to various systemic
290 treatments. We also wanted to reproduce the first 24 or 48 hours of treatment during which antibiotic
291 susceptibility pattern is not determined and clinicians have to deal with a Gram-positive healthcare-
292 associated bloodstream infection.

293 Ethanol alone or in combination is reported to be effective against *in vitro* biofilms as well as
294 decreasing CRBSI and the need for catheter replacement in clinical trials (21, 23, 32). However, under
295 our experimental conditions, it was able to reduce the biofilm but could not completely eradicate it
296 even after 5 consecutive ALT replacements. This suggests that reducing the bacterial load in the
297 TIVAP might suffice for weakening bacterial biofilms, thus enabling the host immune system or
298 systemic antibiotics to control CRBSI, as shown in clinical studies using ethanol. While treatments that

299 do not completely eradicate biofilms efficiently reduce the incidence of CRBSI, therapy such as
300 gentamicin-EDTA might totally eradicate biofilms colonizing the catheters, and would thus have
301 greater potential for curing biofilm-related infections.

302 Recurrence of biofilm-associated infections due to the presence of highly antibiotic tolerant bacteria
303 within biofilms is one of the major challenges for catheter management in the clinical setting (12, 25,
304 26). The existence of such highly tolerant biofilm bacteria was demonstrated in our model by the fact
305 that they could sustain very high concentrations (up to 1,700X MIC) of gentamicin. Gentamicin-EDTA
306 proved to be a potential lock solution able to cure these highly tolerant biofilms and eradicate
307 persistent bacteria, thereby preventing recurrence of Gram-positive as well as Gram-negative (see
308 below) bacterial biofilms on TIVAP. Moreover, currently proposed ALT regimens are used for up to 14
309 days, resulting in reduced access to the device, which could cause distress in patients with limited
310 venous access (30). Development of a rapid and efficient ALT would enable earlier access to the long-
311 term catheter, improving patient outcome. We showed that a single instillation of gentamicin-EDTA
312 ALT, left to dwell for 7 days, effectively eradicated biofilms formed by MSSA and *S. epidermidis*, thus
313 probably reducing the possibility of recurrence. It is noteworthy that the MRSA strain used in this study
314 is gentamicin-resistant, like 3% of MSSA and 11% of MRSA in hospital-acquired *S. aureus* infections
315 in Texas (22). Nevertheless, use of a 5-day ALT regimen led to total eradication of *in vivo* TIVAP-
316 associated MRSA biofilms, suggesting that the gentamicin-EDTA lock solution could still be used in
317 this setting. As EDTA was earlier shown to disrupt biofilm through metal chelation, we speculate that
318 the bacteria released would be more susceptible to the direct antibacterial effect of EDTA (see Table
319 1) (34). Besides, free-swimming bacteria may have been killed by gentamicin that still could have
320 some efficacy at such a high concentration.

321 A reduction in the catheter-associated bacterial biofilm load may not be sufficient to cure CRBSI in
322 patients with impaired immune systems (45). In our *in vivo* study, a single instillation of the gentamicin-
323 EDTA lock was effective at curing immunosuppressed rats with TIVAP-related MSSA infection, in
324 conjunction with vancomycin systemic treatment. Rapid clearance of the pathogen is of clinical
325 importance, especially in immunocompromised and critically ill patients. Thus, gentamicin-EDTA might,
326 in the future, prove to be of great value in clinical settings.

327 Furthermore, we demonstrated the efficacy of the gentamicin-EDTA antibiotic lock solution against
328 biofilms of *E. coli* and *P. aeruginosa* pathogenic strains. Although multiresistance associated with

329 Gram-negative pathogens is of some concern, these bacteria are often overlooked in studies using
330 catheter-related infections (8, 20, 42). In the case of *P. aeruginosa* CRBSI, treatment failures are
331 frequent and conservative management is often excluded (30). Gentamicin-EDTA could be an
332 excellent candidate lock for such patients, as it extends the possibility of conservative therapy.

333 In summary, we conclude that gentamicin-EDTA effectively eradicated the *in vivo* TIVAP-associated
334 biofilms of all tested strains. Gentamicin and ethanol were also effective at reducing MSSA biofilm, but
335 were not able to completely eradicate the biofilm from implanted TIVAP. In light of these results, we
336 believe that the gentamicin-EDTA lock deserves further exploration for use in clinical practice.

337

338

339 **ACKNOWLEDGMENTS**

340 We are grateful to Brigitte Arbeille and Claude Lebos (LBCME, Faculté de Médecine de Tours) for
341 their help in performing electronic microscopy. This work was supported by grants from the Institut
342 Pasteur and, in part, from the Institut Mérieux-Institut Pasteur collaborative research program. D.L.
343 was supported by a grant from the AXA Research Fund.

344

345

346

347
348

REFERENCES

349
350

- 351 1. **Ahmad, N. M., and A. D. Rojzman.** 2010. Successful treatment of daptomycin-nonsusceptible
352 methicillin-resistant *Staphylococcus aureus* bacteremia with the addition of rifampin to
353 daptomycin. *Ann Pharmacother* **44**:918-921.
- 354 2. **Allon, M.** 2007. Current management of vascular access. *Clin J Am Soc Nephrol* **2**:786-800.
- 355 3. **Betjes, M. G., and M. van Agteren.** 2004. Prevention of dialysis catheter-related sepsis with
356 a citrate-taurolidine-containing lock solution. *Nephrol Dial Transplant* **19**:1546-1551.
- 357 4. **Bleyer, A. J., L. Mason, G. Russell, Raad, II, and R. J. Sherertz.** 2005. A randomized,
358 controlled trial of a new vascular catheter flush solution (minocycline-EDTA) in temporary
359 hemodialysis access. *Infect Control Hosp Epidemiol* **26**:520-524.
- 360 5. **Blot, S. I., P. Depuydt, L. Annemans, D. Benoit, E. Hoste, J. J. De Waele, J.**
361 **Decruyenaere, D. Vogelaers, F. Colardyn, and K. H. Vandewoude.** 2005. Clinical and
362 economic outcomes in critically ill patients with nosocomial catheter-related bloodstream
363 infections. *Clin Infect Dis* **41**:1591-1598.
- 364 6. **Bookstaver, P. B., J. C. Williamson, B. K. Tucker, Raad, II, and R. J. Sherertz.** 2009.
365 Activity of novel antibiotic lock solutions in a model against isolates of catheter-related
366 bloodstream infections. *Ann Pharmacother* **43**:210-219.
- 367 7. **Carratala, J., J. Niubo, A. Fernandez-Sevilla, E. Juve, X. Castellsague, J. Berlanga, J.**
368 **Linares, and F. Gudiol.** 1999. Randomized, double-blind trial of an antibiotic-lock technique
369 for prevention of gram-positive central venous catheter-related infection in neutropenic
370 patients with cancer. *Antimicrob Agents Chemother* **43**:2200-2204.
- 371 8. **Chang, L., J. S. Tsai, S. J. Huang, and C. C. Shih.** 2003. Evaluation of infectious
372 complications of the implantable venous access system in a general oncologic population. *Am*
373 *J Infect Control* **31**:34-39.
- 374 9. **Chauhan, A., D. Lebeaux, B. Decante, I. Kriegel, M. C. Escande, J. M. Ghigo, and C.**
375 **Beloin.** 2012. A rat model of central venous catheter to study establishment of long-term
376 bacterial biofilm and related acute and chronic infections. *PLoS One* **7**:e37281.

- 377 10. **Console, G., C. Calabro, P. Nardulli, F. Diguseppe, A. Rucci, P. Russo, and P. Geppetti.**
378 2007. Clinical and economic effects of central venous catheters on oncology patient care. *J*
379 *Chemother* **19**:309-314.
- 380 11. **Dannenberg, C., U. Bierbach, A. Rothe, J. Beer, and D. Korholz.** 2003. Ethanol-lock
381 technique in the treatment of bloodstream infections in pediatric oncology patients with broviac
382 catheter. *J Pediatr Hematol Oncol* **25**:616-621.
- 383 12. **Darouiche, R. O.** 2004. Treatment of infections associated with surgical implants. *N Engl J*
384 *Med* **350**:1422-1429.
- 385 13. **Del Pozo, J. L., M. Garcia Cenoz, S. Hernaez, A. Martinez, A. Serrera, A. Aguinaga, M.**
386 **Alonso, and J. Leiva.** 2009. Effectiveness of teicoplanin versus vancomycin lock therapy in
387 the treatment of port-related coagulase-negative staphylococci bacteraemia: a prospective
388 case-series analysis. *Int J Antimicrob Agents* **34**:482-485.
- 389 14. **Donlan, R. M.** 2002. Biofilms: microbial life on surfaces. *Emerging Infect Dis* **8**:881-890.
- 390 15. **Droste, J. C., H. A. Jeraj, A. MacDonald, and K. Farrington.** 2003. Stability and *in vitro*
391 efficacy of antibiotic-heparin lock solutions potentially useful for treatment of central venous
392 catheter-related sepsis. *J Antimicrob Chemother* **51**:849-855.
- 393 16. **Fernandez-Hidalgo, N., B. Almirante, R. Calleja, I. Ruiz, A. M. Planes, D. Rodriguez, C.**
394 **Pigrau, and A. Pahissa.** 2006. Antibiotic-lock therapy for long-term intravascular catheter-
395 related bacteraemia: results of an open, non-comparative study, p. 1172-1180, *J Antimicrob*
396 *Chemother*, vol. 57.
- 397 17. **Fortun, J., F. Grill, P. Martin-Davila, J. Blazquez, M. Tato, J. Sanchez-Corral, L. Garcia-**
398 **San Miguel, and S. Moreno.** 2006. Treatment of long-term intravascular catheter-related
399 bacteraemia with antibiotic-lock therapy. *J Antimicrob Chemother* **58**:816-821.
- 400 18. **Foucault, M.-L., L. Thomas, S. Goussard, B. R. Branchini, and C. Grillo-Courvalin.** 2010.
401 *In vivo* bioluminescence imaging for the study of intestinal colonization by *Escherichia coli* in
402 mice. *Applied and Environmental Microbiology* **76**:264-274.
- 403 19. **Fowler, V. G., Jr., A. Justice, C. Moore, D. K. Benjamin, Jr., C. W. Woods, S. Campbell, L.**
404 **B. Reller, G. R. Corey, N. P. Day, and S. J. Peacock.** 2005. Risk factors for hematogenous
405 complications of intravascular catheter-associated *Staphylococcus aureus* bacteremia. *Clin*
406 *Infect Dis* **40**:695-703.

- 407 20. **Funalleras, G., N. Fernandez-Hidalgo, A. Borrego, B. Almirante, A. M. Planes, D.**
408 **Rodriguez, I. Ruiz, and A. Pahissa.** 2011. Effectiveness of antibiotic-lock therapy for long-
409 term catheter-related bacteremia due to Gram-negative bacilli: a prospective observational
410 study. *Clin Infect Dis* **53**:e129-132.
- 411 21. **Ghannoum, M. A., N. Isham, and M. R. Jacobs.** 2011. Antimicrobial activity of B-Lock
412 against bacterial and *Candida* spp. causing catheter-related bloodstream infections.
413 *Antimicrob Agents Chemother* **55**:4430-4431.
- 414 22. **Hulten, K. G., S. L. Kaplan, L. B. Lamberth, K. Slimp, W. A. Hammerman, M. Carrillo-**
415 **Marquez, J. R. Starke, J. Versalovic, and E. O. Mason, Jr.** 2009. Hospital-acquired
416 *Staphylococcus aureus* infections at Texas Children's Hospital, 2001-2007. *Infect Control*
417 *Hosp Epidemiol* **31**:183-190.
- 418 23. **Jones, B. A., M. A. Hull, D. S. Richardson, D. Zurakowski, K. Gura, S. C. Fitzgibbons, D.**
419 **Duro, C. W. Lo, C. Duggan, and T. Jaksic.** 2010. Efficacy of ethanol locks in reducing
420 central venous catheter infections in pediatric patients with intestinal failure. *J Pediatr Surg*
421 **45**:1287-1293.
- 422 24. **Lafrance JP, Iqbal S, Lelorier J, Dasgupta K, Ritchie J, Ward L, Benaroya S, Barré P,**
423 **Cantarovich M, Ghannoum M, Proulx N, Vasilevsky M, and R. E.** 2010. Vascular access-
424 related bloodstream infections in First Nations, community and teaching Canadian dialysis
425 units, and other centre-level predictors. *Nephrology Clinical Practices* **114**:c204-214.
- 426 25. **Lewis, K.** 2007. Persister cells, dormancy and infectious disease. *Nat Rev Microbiol* **5**:48-56.
- 427 26. **Lewis, K.** 2001. Riddle of biofilm resistance. *Antimicrob Agents Chemother* **45**:999-1007.
- 428 27. **Mack, D., A. Sabottke, S. Dobinsky, H. Rohde, M. A. Horstkotte, and J. K. Knobloch.**
429 2002. Differential expression of methicillin resistance by different biofilm-negative
430 *Staphylococcus epidermidis* transposon mutant classes. *Antimicrob Agents Chemother*
431 **46**:178-183.
- 432 28. **Maki, D. G., D. M. Kluger, and C. J. Crnich.** 2006. The risk of bloodstream infection in adults
433 with different intravascular devices: a systematic review of 200 published prospective studies.
434 *Mayo Clin Proc* **81**:1159-1171.
- 435 29. **McIntyre, C. W., L. J. Hulme, M. Taal, and R. J. Fluck.** 2004. Locking of tunneled
436 hemodialysis catheters with gentamicin and heparin. *Kidney Int* **66**:801-805.

- 437 30. **Mermel, Leonard A., M. Allon, E. Bouza, Donald E. Craven, P. Flynn, Naomi P. O'grady,**
438 **Issam I. Raad, Bart J. A. Rijnders, Robert J. Sherertz, and David K. Warren.** 2009. Clinical
439 Practice Guidelines for the Diagnosis and Management of Intravascular Catheter-Related
440 Infection: 2009 Update by the Infectious Diseases Society of America. *CLIN INFECT DIS*
441 **49:1-45.**
- 442 31. **O'Grady, N. P.** 2002. Applying the science to the prevention of catheter-related infections. *J*
443 *Crit Care* **17:114-121.**
- 444 32. **Oliveira, C., A. Nasr, M. Brindle, and P. W. Wales.** 2012. Ethanol locks to prevent catheter-
445 related bloodstream infections in parenteral nutrition: a meta-analysis. *Pediatrics* **129:318-329.**
- 446 33. **Perra-Ruiz, J., C. Vidailac, W. E. Rose, and M. J. Rybak.** 2010. Activities of high-dose
447 daptomycin, vancomycin, and moxifloxacin alone or in combination with clarithromycin or
448 rifampin in a novel *in vitro* model of *Staphylococcus aureus* biofilm. *Antimicrob Agents*
449 *Chemother* **54:4329-4334.**
- 450 34. **Raad, Il, X. Fang, X. M. Keutgen, Y. Jiang, R. Sherertz, and R. Hachem.** 2008. The role of
451 chelators in preventing biofilm formation and catheter-related bloodstream infections. *Curr*
452 *Opin Infect Dis* **21:385-392.**
- 453 35. **Raad, Il, R. Y. Hachem, H. A. Hanna, X. Fang, Y. Jiang, T. Dvorak, R. J. Sherertz, and D.**
454 **P. Kontoyiannis.** 2008. Role of ethylene diamine tetra-acetic acid (EDTA) in catheter lock
455 solutions: EDTA enhances the antifungal activity of amphotericin B lipid complex against
456 *Candida* embedded in biofilm. *Int J Antimicrob Agents* **32:515-518.**
- 457 36. **Raad, Il, M. Luna, S. A. Khalil, J. W. Costerton, C. Lam, and G. P. Bodey.** 1994. The
458 relationship between the thrombotic and infectious complications of central venous catheters.
459 *JAMA* **271:1014-1016.**
- 460 37. **Raad, I.** 1998. Intravascular-catheter-related infections. *Lancet* **351:893-898.**
- 461 38. **Raad, I., H. Hanna, T. Dvorak, G. Chaiban, and R. Hachem.** 2007. Optimal antimicrobial
462 catheter lock solution, using different combinations of minocycline, EDTA, and 25-percent
463 ethanol, rapidly eradicates organisms embedded in biofilm. *Antimicrob Agents Chemother*
464 **51:78-83.**

- 465 39. **Ramphal, R., V. Balloy, J. Jyot, A. Verma, M. Si-Tahar, and M. Chignard.** 2008. Control of
466 *Pseudomonas aeruginosa* in the lung requires the recognition of either lipopolysaccharide or
467 flagellin. *J Immunol* **181**:586-592.
- 468 40. **Rijnders, B. J., E. Van Wijngaerden, S. J. Vandecasteele, M. Stas, and W. E. Peetermans.**
469 2005. Treatment of long-term intravascular catheter-related bacteraemia with antibiotic lock:
470 randomized, placebo-controlled trial. *J Antimicrob Chemother* **55**:90-94.
- 471 41. **Scott, R.** 2009. The Direct Medical Costs of Healthcare-Associated Infections in U.S.
472 Hospitals and the Benefits of Prevention.1-16.
- 473 42. **Seifert, H.** 1997. Catheter-related Infections. Catheter-related infections due to gram-negative
474 bacilli. In: Seifert H, Jansen B, Farr BM, editors. **New York: Marcel Dekker**:255-284.
- 475 43. **Shanks, R. M., J. L. Sargent, R. M. Martinez, M. L. Graber, and G. A. O'Toole.** 2006.
476 Catheter lock solutions influence staphylococcal biofilm formation on abiotic surfaces. *Nephrol*
477 *Dial Transplant* **21**:2247-2255.
- 478 44. **Stewart, P. S., and J. W. Costerton.** 2001. Antibiotic resistance of bacteria in biofilms. *The*
479 *Lancet* **358**:135-138.
- 480 45. **Sydnor, E. R., and T. M. Perl.** 2011. Hospital epidemiology and infection control in acute-care
481 settings. *Clin Microbiol Rev* **24**:141-173.
- 482 46. **Timsit, J. F., Y. Dubois, C. Minet, A. Bonadona, M. Lugosi, C. Ara-Somohano, R.**
483 **Hamidfar-Roy, and C. Schwebel.** 2011. New challenges in the diagnosis, management, and
484 prevention of central venous catheter-related infections. *Semin Respir Crit Care Med* **32**:139-
485 150.
- 486 47. **Tyler, L. S., T. L. Rehder, and R. B. Davis.** 1981. Effect of gentamicin on heparin activity.
487 *Am J Hosp Pharm* **38**:537-540.
- 488 48. **Van Praagh, A. D., T. Li, S. Zhang, A. Arya, L. Chen, X. X. Zhang, S. Bertolami, and L. I.**
489 **Mortin.** 2011. Daptomycin antibiotic lock therapy in a rat model of staphylococcal central
490 venous catheter biofilm infections. *Antimicrob Agents Chemother* **55**:4081-4089.
- 491 49. **Vandijck, D. M., M. Depaemelaere, S. O. Labeau, P. O. Depuydt, L. Annemans, F. M.**
492 **Buyle, S. Oeyen, K. E. Colpaert, R. P. Peleman, S. I. Blot, and J. M. Decruyenaere.** 2008.
493 Daily cost of antimicrobial therapy in patients with Intensive Care Unit-acquired, laboratory-
494 confirmed bloodstream infection. *Int J Antimicrob Agents* **31**:161-165.

- 495 50. **Venkatesh, M., L. Rong, I. Raad, and J. Versalovic.** 2009. Novel synergistic antibiofilm
496 combinations for salvage of infected catheters. *J Med Microbiol* **58**:936-944.
- 497 51. **Vescia, S., A. K. Baumgärtner, V. R. Jacobs, M. Kiechle-Bahat, A. Rody, S. Loibl, and N.**
498 **Harbeck.** 2008. Management of venous port systems in oncology: a review of current
499 evidence. *Ann Oncol* **19**:9-15.
- 500 52. **Vuong, C., S. Kocianova, J. Yu, Jagath L. Kadurugamuwa, and M. Otto.** 2008.
501 Development of Real-Time *In Vivo* Imaging of Device-Related *Staphylococcus epidermidis*
502 Infection in Mice and Influence of Animal Immune Status on Susceptibility to Infection. *J Infect*
503 *Dis* **198**:258-261.
- 504 53. **Weijmer, M. C., M. A. van den Dorpel, P. J. Van de Ven, P. M. ter Wee, J. A. van Geelen,**
505 **J. O. Groeneveld, B. C. van Jaarsveld, M. G. Koopmans, C. Y. le Poole, A. M. Schrande-**
506 **Van der Meer, C. E. Siegert, and K. J. Stas.** 2005. Randomized, clinical trial comparison of
507 trisodium citrate 30% and heparin as catheter-locking solution in hemodialysis patients. *J Am*
508 *Soc Nephrol* **16**:2769-2777.
- 509 54. **Zuluaga, A. F., M. Agudelo, J. J. Cardeno, C. A. Rodriguez, and O. Vesga.** 2010.
510 Determination of therapeutic equivalence of generic products of gentamicin in the neutropenic
511 mouse thigh infection model. *PLoS one* **5**:e10744.
- 512
- 513
- 514

515 **TABLE:**

516

517 **Table 1.** MIC and concentrations used for *in vivo* ALT. ALT: antibiotic lock therapy; EDTA: ethylenediamine-tetraacetic acid; MIC: minimal inhibitory
518 concentration; MSSA: methicillin-susceptible *S. aureus*; MRSA: methicillin-resistant *S. aureus*; ND: not done. MIC results are expressed as mean of at
519 least 3 experiments.

520

Microorganism	MIC					ALT		
	Gentamicin ($\mu\text{g/mL}$)	Vancomycin ($\mu\text{g/mL}$)	EDTA (mg/mL)	Ethanol (%)		Gentamicin ($\mu\text{g/mL}$)	EDTA (mg/mL)	Ethanol (%)
<i>MSSA</i>	8	1.5	0.94	6.25		5,000	30	70
<i>MRSA</i>	>1,024	1.5	0.94	12.5		5,000	30	ND
<i>S. epidermidis</i>	3	3	0.94	12.5		5,000	30	ND
<i>E. coli</i>	8	ND	3.75	12.5		5,000	30	ND
<i>P. aeruginosa</i>	4	ND	3.75	6.25		5,000	30	ND

521 **FIGURE LEGENDS**

522

523 **Figure 1. Gentamicin-EDTA ALT completely eradicates MSSA biofilm from the imp**

524 ALT was instilled in TIVAP of immunocompetent rats (day 0) and was associated
525 vancomycin to treat MSSA biofilm colonization (number (n) of rats used for each treatm
526 was renewed every 24 h for 5 days and its efficacy was monitored as photon emissions
527 a representative animal are shown. (A) Control rats with PBS ALT. (B) 70% ethanol ALT
528 gentamicin ALT. (D) 30 mg/mL EDTA alone. (E) Combined gentamicin (5 mg/mL) a
529 mg/mL) ALT. In (A) to (E) representative experiments are shown. (F) Rats were sacrifice
530 of treatment, TIVAP was harvested and monitored for photon emissions. (G) Bacte
531 TIVAP were harvested and plated on TSB agar for CFU/mL. Results for CFU are mea
532 deviation. Statistical analysis was done using one-way analysis of variance (ANOVA) \n
533 Prism version 5.0c. p-value < 0.05 considered significant, ** (p ≤ 0.009).

534

535 **Figure 2. Gentamicin-EDTA ALT reduces time to eradicate MSSA biofilm *in vivo*.**

536 EDTA lock solution was instilled in MSSA-colonized TIVAP of immunocompetent
537 conjunction with systemic vancomycin treatment. Rats were sacrificed either 1 day or i
538 after a single instillation and monitored as photon emissions. Results from a representat
539 shown. Bacterial cells were harvested from the TIVAP on respective days (day 1 or day
540 for CFU/mL. (A) Control rats with PBS ALT. (B) TIVAP harvested from control rats. (C
541 EDTA-instilled rats. (D) TIVAP harvested from gentamicin-EDTA-treated rats. (E) |
542 harvested from TIVAP and plated on TSB agar for CFU/mL. Eradication of *in vivo* TIV,
543 MSSA biofilm was confirmed by scanning electron microscopy (SEM). (F) Gentamic
544 TIVAP. (G) EDTA-ALT-treated TIVAP. (H) Gentamicin-EDTA-ALT-treated TIVAP. Resul
545 mean +/- standard deviation. Statistical analysis was done using one-way analysi
546 (ANOVA) with Graphpad Prism version 5.0c. p-Value < 0.05 considered significant, **
547 (p≤ 0.09).

548

549

550 **Figure 3. Gentamicin-EDTA/ALT eradicates MSSA TIVAP-associated biofilm in**
551 **immunosuppressed rats.** TIVAP-implanted and immunosuppressed rats (n= 3 for each treatment)
552 were contaminated with MSSA and allowed to form biofilm for 3 days prior to ALT instillation and
553 vancomycin systemic antibiotic injection. Treatment efficacy was monitored as photon emissions.
554 Results from a representative animal are shown. (A) Control rats with PBS ALT. (B) TIVAP harvested
555 from control rats. (C) Gentamicin-alone-instilled rats. (D) TIVAP harvested from gentamicin-alone-
556 instilled rats. (E) Gentamicin-EDTA-instilled rats. (F) TIVAP harvested from gentamicin-EDTA-instilled
557 rats. (G) Bacteria were harvested from TIVAP and plated on TSB agar for CFU/mL. Genta: gentamicin.
558 Results for CFU are mean +/- standard deviation. Statistical analysis was done using one-way
559 analysis of variance (ANOVA) with Graphpad Prism version 5.0c. p-Value < 0.05 considered
560 significant, ** (p ≤ 0.003).

561

562 **Figure 4. Gentamicin-EDTA ALT eradicates *S. epidermidis* and MRSA biofilms.** Three-day-old *S.*
563 *epidermidis* or MRSA TIVAP-associated biofilm in immunocompetent rat was treated by a 1-day or a
564 5-day regimen of gentamicin-EDTA ALT in conjunction with systemic vancomycin (number of animals:
565 n=3 for each treatment). Rats were sacrificed 8 days post-ALT, TIVAP was removed aseptically and
566 harvested cells were plated on TSB agar plates for CFU/mL. Results for CFU are mean +/- standard
567 deviation. Statistical analysis was done using one-way analysis of variance (ANOVA) with Graphpad
568 Prism version 5.0c. p-value < 0.05 considered significant, * (p ≤ 0.02).

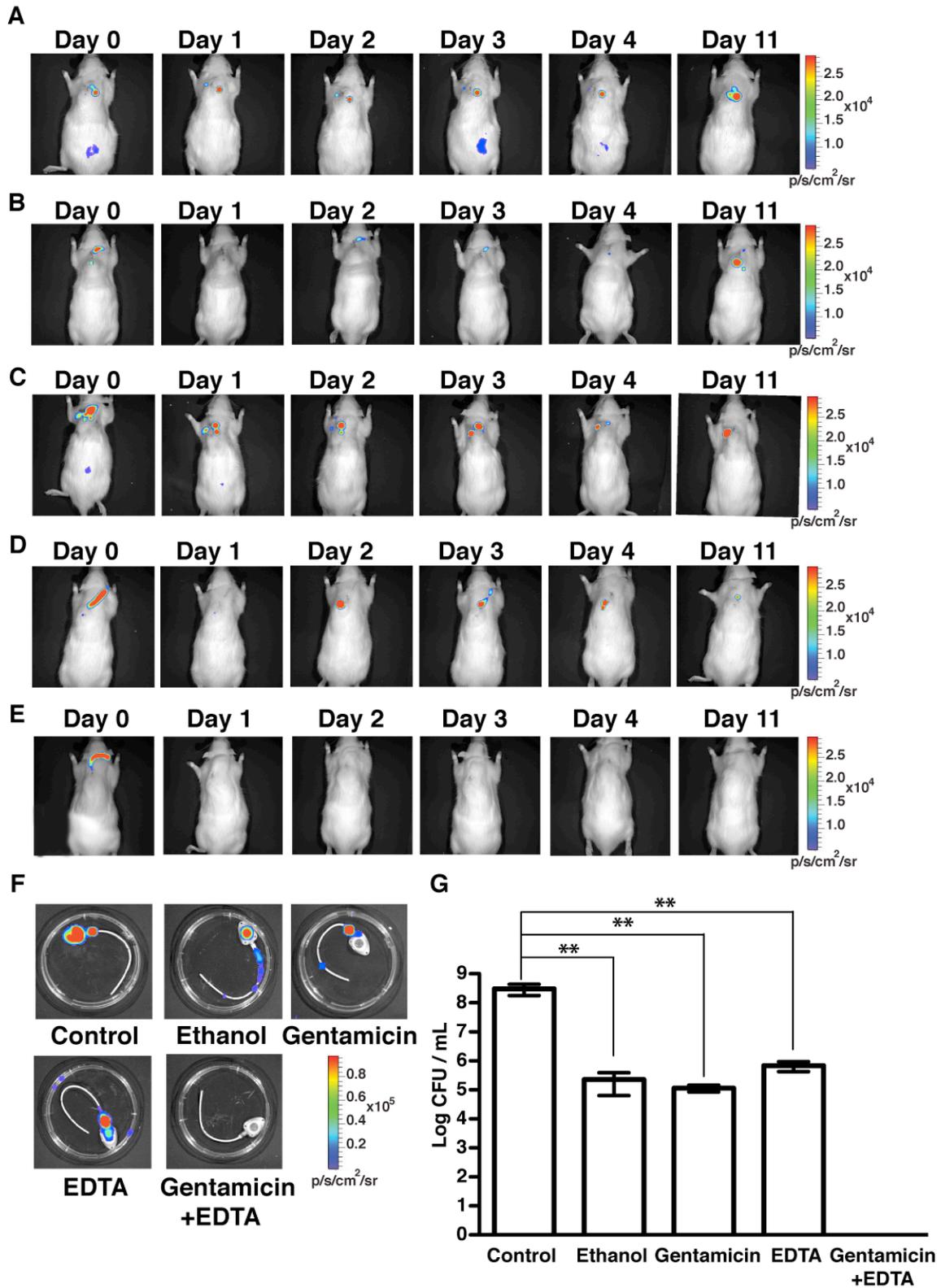
569

570 **Figure 5. The gentamicin-EDTA lock solution is also effective against Gram-negative bacteria.**
571 Three-day-old *E. coli* or *P. aeruginosa* TIVAP-associated biofilm in immunocompetent rats was treated
572 by a 1-day regimen of gentamicin-EDTA ALT in conjunction with systemic gentamicin (number of
573 animals: n=3 for each treatment). Treatment efficacy was monitored as photon emissions. Results
574 from a representative animal are shown. (A) TIVAP-implanted control rats (with PBS ALT) with *E. coli*
575 colonization. (B) *E. coli*-colonized TIVAP-implanted rats with gentamicin-EDTA ALT. (C) TIVAP from
576 control rats with *E. coli* colonization. (D) TIVAP harvested from *E. coli*-colonized and gentamicin-
577 EDTA-instilled rats. (E) TIVAP-implanted control rats (with PBS ALT) with *P. aeruginosa* colonization.
578 (F) *P. aeruginosa*-colonized TIVAP-implanted rats with gentamicin-EDTA ALT. (G) TIVAP from control
579 rats with *P. aeruginosa* colonization. (H) TIVAP harvested from *P. aeruginosa*-colonized and

580 gentamicin-EDTA-instilled rats. (l) Rats were sacrificed 8 days post-ALT, TIVAP were aseptically
581 removed and harvested cells were plated on LB agar (*E. coli* or *P. aeruginosa*) plates for CFU/mL.
582 Genta: gentamicin. Results for CFU are mean +/- standard deviation. Statistical analysis was done
583 using one-way analysis of variance (ANOVA) with Graphpad Prism version 5.0c. p-Value < 0.05
584 considered significant, *** (p < 0.0001), ** (p < 0.001) and * (p < 0.01).

585
586

587 **FIGURES**

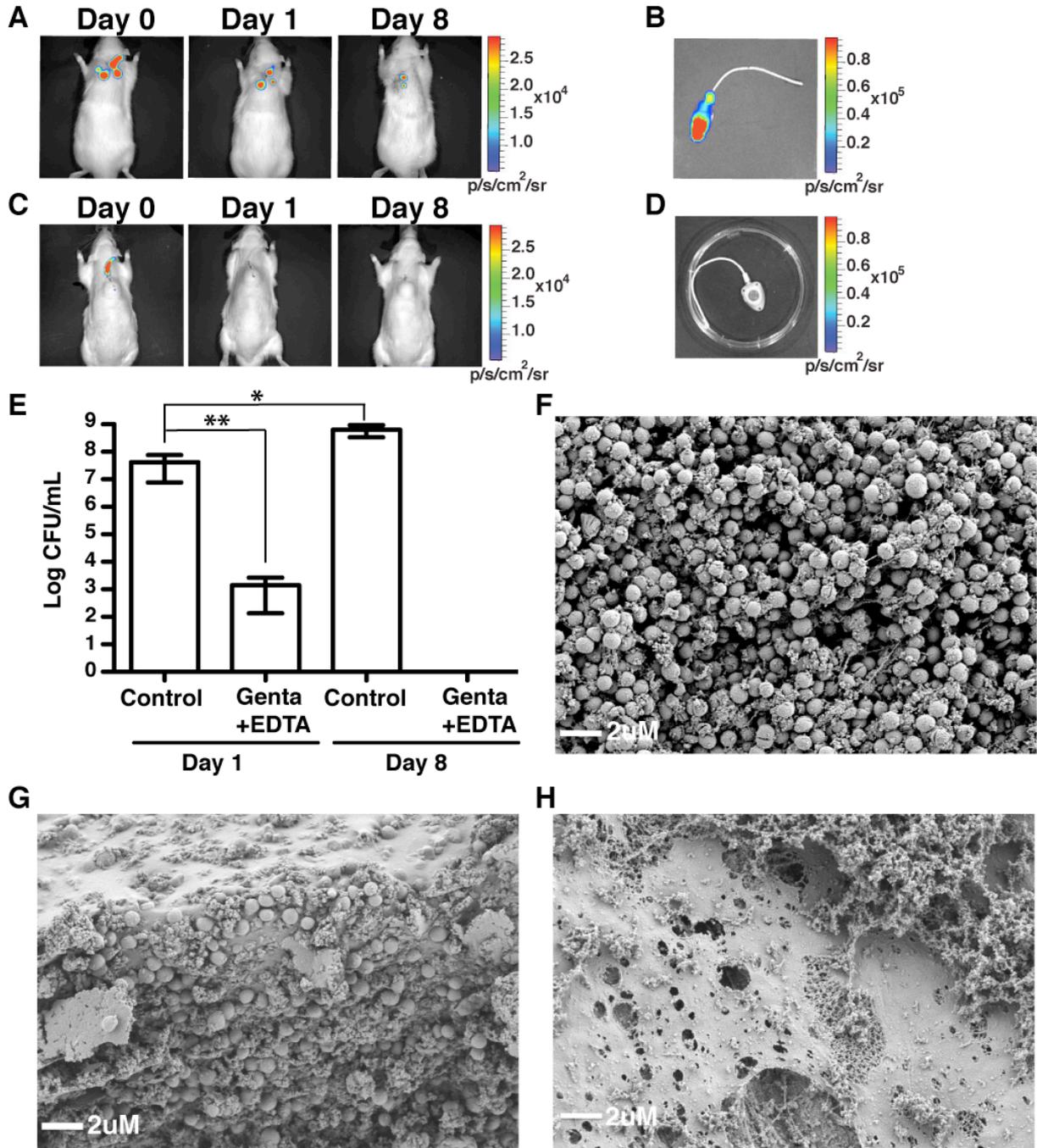


588

589 **Figure 1.** Chauhan *et al.*

590

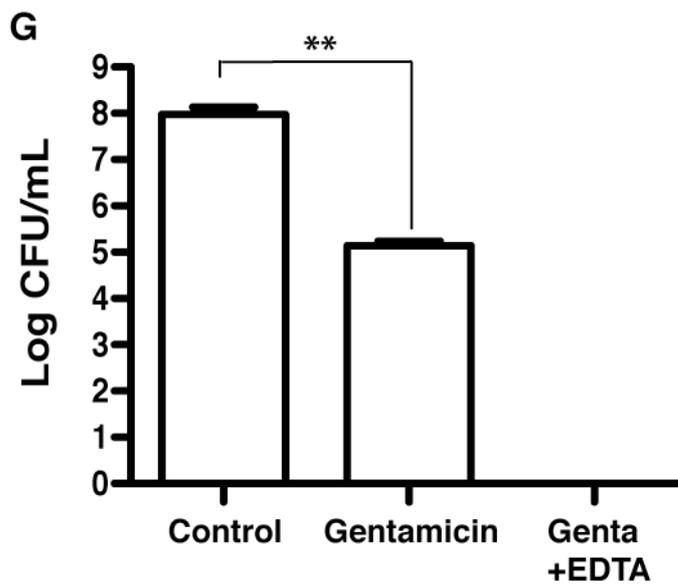
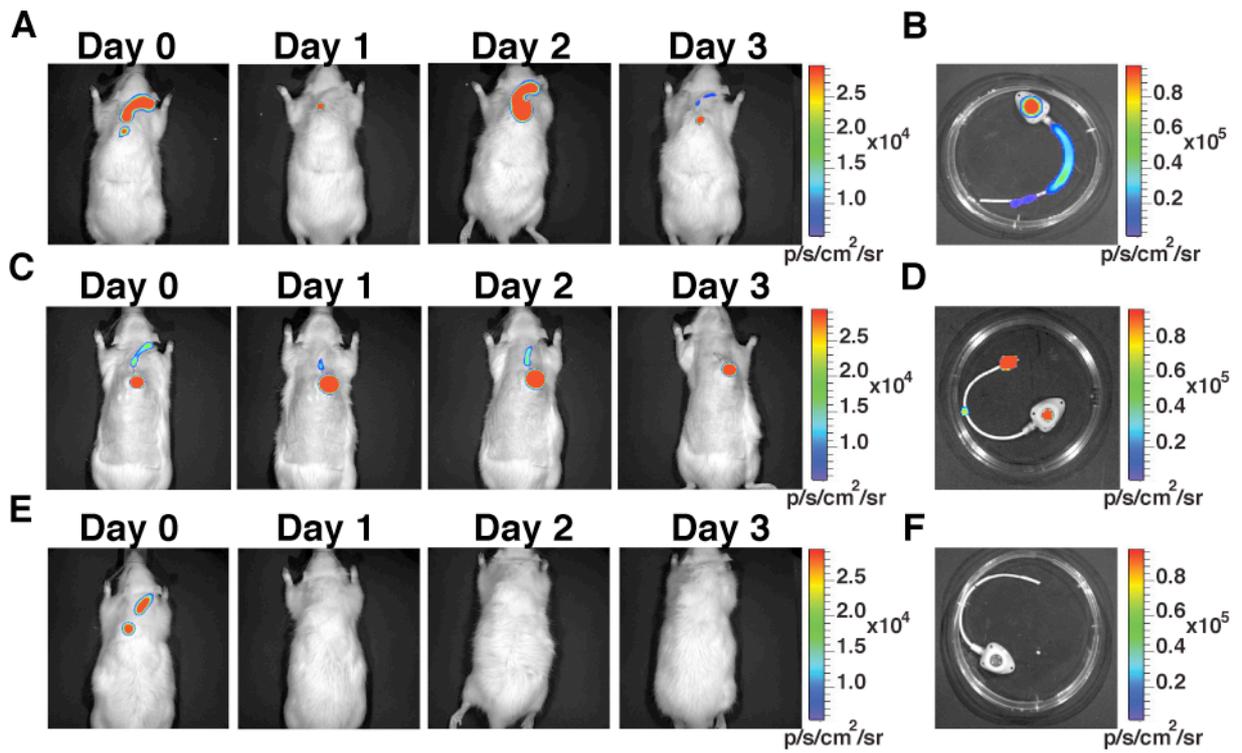
591



592

593 **Figure 2.** Chauhan *et al.*

594

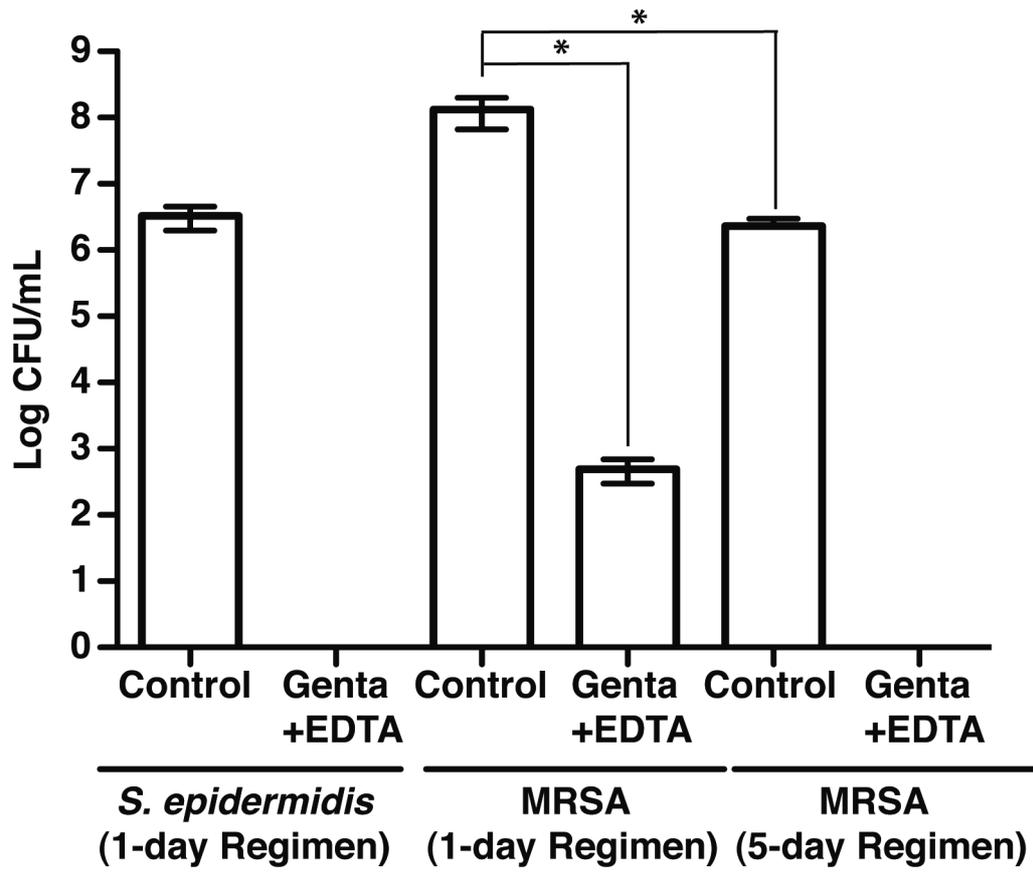


595

596 **Figure 3.** Chauhan *et al.*

597

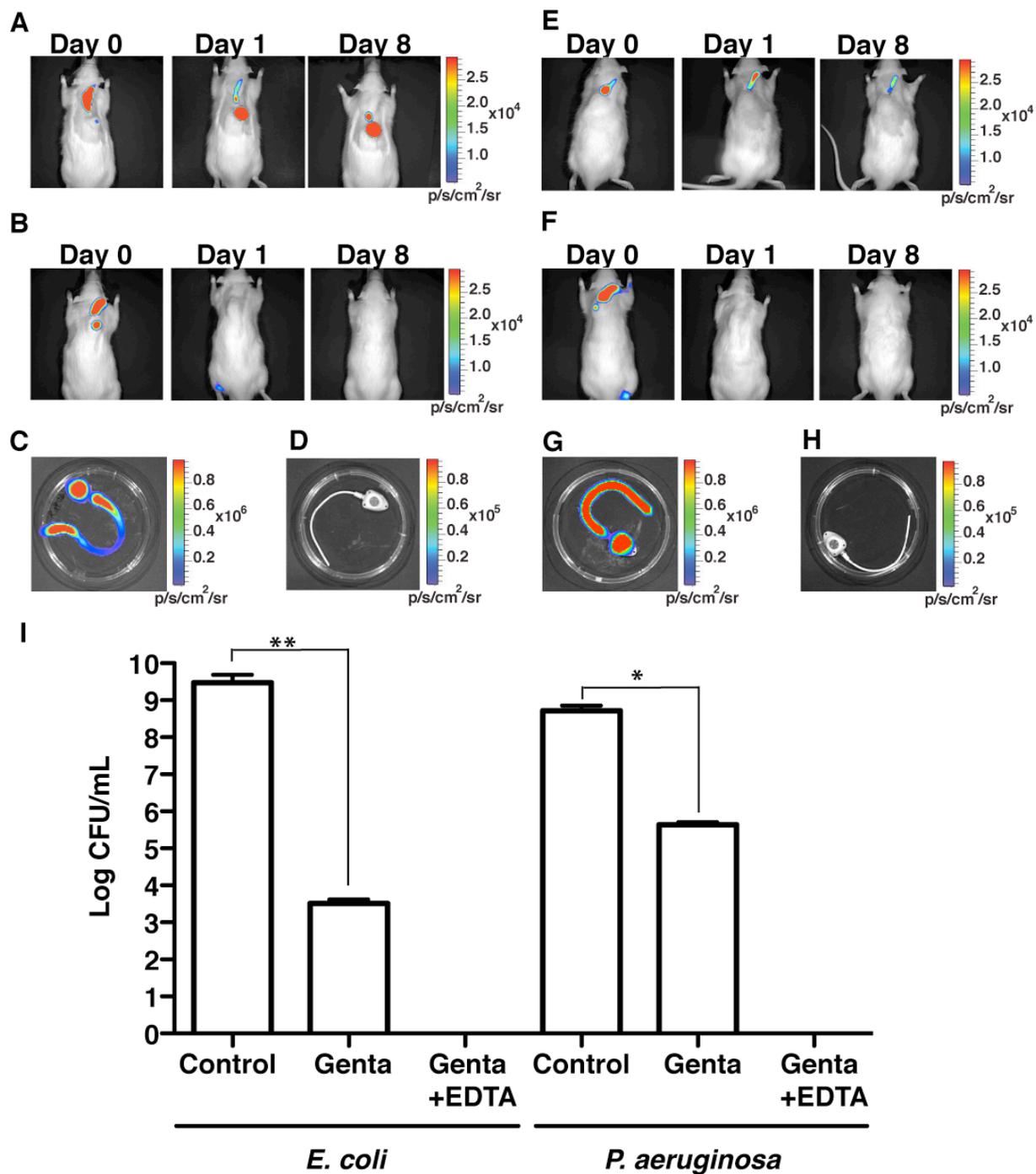
598



599
600

601 **Figure 4.** Chauhan *et al.*

602



603
604 **Figure 5.** Chauhan *et al.*

605