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

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

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.

INTRODUCTION

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

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

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

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

MATERIALS AND METHODS

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

Determination of* in vitro *minimal inhibitory concentrations (MICs). MICs were determined by broth microdilution as per CLSI (Methods for dilution of antimicrobial susceptibility tests for bacteria that grow aerobically; Approved Standard, Eighth Edition. CLSI Document M07-A8. Wayne, PA: Clinical and Laboratory Standards Institute; 2009). Briefly, exponentially growing bacteria were diluted to a final inoculum of $5x10^5$ bacteria/mL. Results were read after 16 to 18 h of culture. MIC was defined as the first well without visible growth. MIC was performed in TSB glucose at 0.25% for *S. aureus* and *S. epidermidis* and in LB for *E. coli* and *P. aeruginosa* (Table 1).

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

Animal model. Male CD/SD (IGS:Crl) rats purchased from Charles River weighed 275-300 g and were allowed to acclimatize using 12 h day/night cycles for one week before use at the Institut Pasteur animal facilities accredited by the French Ministry of Agriculture to perform experiments on live rodents (accreditation #A75-15 27, issued on November 12, 2004 and #A75-15 04, issued on May 22, 2008), in compliance with French and European regulations on care and protection of laboratory animals (EC Directive 86/609, French Law 2001-486 issued on June 6, 2001). Protocols were approved by the veterinary staff of the Institut Pasteur animal facility and were performed in compliance with NIH 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 µL 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 µL 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 µL 1X PBS
146 was used for TIVAP inoculation and confirmed by plating for CFU/mL. Control catheterized and
147 immunosuppressed rats received 100 µL 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

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

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

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

RESULTS

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

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

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

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

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

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

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

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

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

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

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

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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TABLE:

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

Microorganism	MIC				ALT		
	Gentamicin (μg/mL)	Vancomycin (μg/mL)	EDTA (mg/mL)	Ethanol (%)	Gentamicin (μg/mL)	EDTA (mg/mL)	Ethanol (%)
MSSA	8	1.5	0.94	6.25	5,000	30	70
MRSA	>1,024	1.5	0.94	12.5	5,000	30	ND
S. epidermidis	3	3	0.94	12.5	5,000	30	ND
E. coli	8	ND	3.75	12.5	5,000	30	ND
P. aeruginosa	4	ND	3.75	6.25	5,000	30	ND

FIGURE LEGENDS

Figure 1. Gentamicin-EDTA ALT completely eradicates MSSA biofilm from the imp
ALT was instilled in TIVAP of immunocompetent rats (day 0) and was associated
vancomycin to treat MSSA biofilm colonization (number (n) of rats used for each treatm
was renewed every 24 h for 5 days and its efficacy was monitored as photon emissions
a representative animal are shown. (A) Control rats with PBS ALT. (B) 70% ethanol ALT
gentamicin ALT. (D) 30 mg/mL EDTA alone. (E) Combined gentamicin (5 mg/mL) a
mg/mL) ALT. In (A) to (E) representative experiments are shown. (F) Rats were sacrifice
of treatment, TIVAP was harvested and monitored for photon emissions. (G) Bacte
TIVAP were harvested and plated on TSB agar for CFU/mL. Results for CFU are mea
deviation. Statistical analysis was done using one-way analysis of variance (ANOVA) v
Prism version 5.0c. p-value < 0.05 considered significant, ** ($p \leq 0.009$).

Figure 2. Gentamicin-EDTA ALT reduces time to eradicate MSSA biofilm *in vivo*.
EDTA lock solution was instilled in MSSA-colonized TIVAP of immunocompetent
conjunction with systemic vancomycin treatment. Rats were sacrificed either 1 day or 7
after a single instillation and monitored as photon emissions. Results from a representat
shown. Bacterial cells were harvested from the TIVAP on respective days (day 1 or day
for CFU/mL. (A) Control rats with PBS ALT. (B) TIVAP harvested from control rats. (C
EDTA-instilled rats. (D) TIVAP harvested from gentamicin-EDTA-treated rats. (E)
harvested from TIVAP and plated on TSB agar for CFU/mL. Eradication of *in vivo* TIV
MSSA biofilm was confirmed by scanning electron microscopy (SEM). (F) Gentamic
TIVAP. (G) EDTA-ALT-treated TIVAP. (H) Gentamicin-EDTA-ALT-treated TIVAP. Resul
mean +/- standard deviation. Statistical analysis was done using one-way analysi
(ANOVA) with Graphpad Prism version 5.0c. p-Value < 0.05 considered significant, **
($p \leq 0.09$).

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

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

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

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

FIGURES

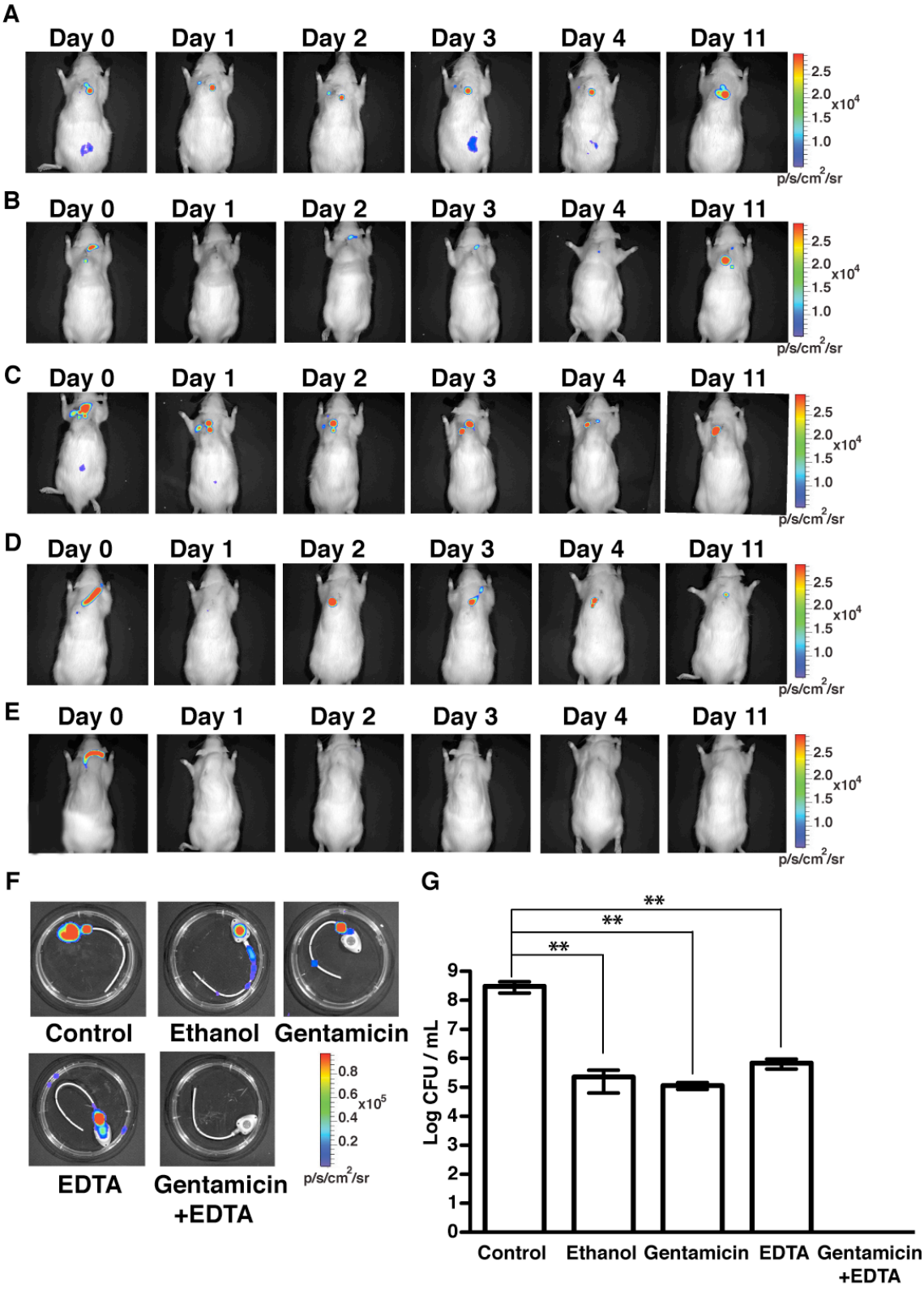
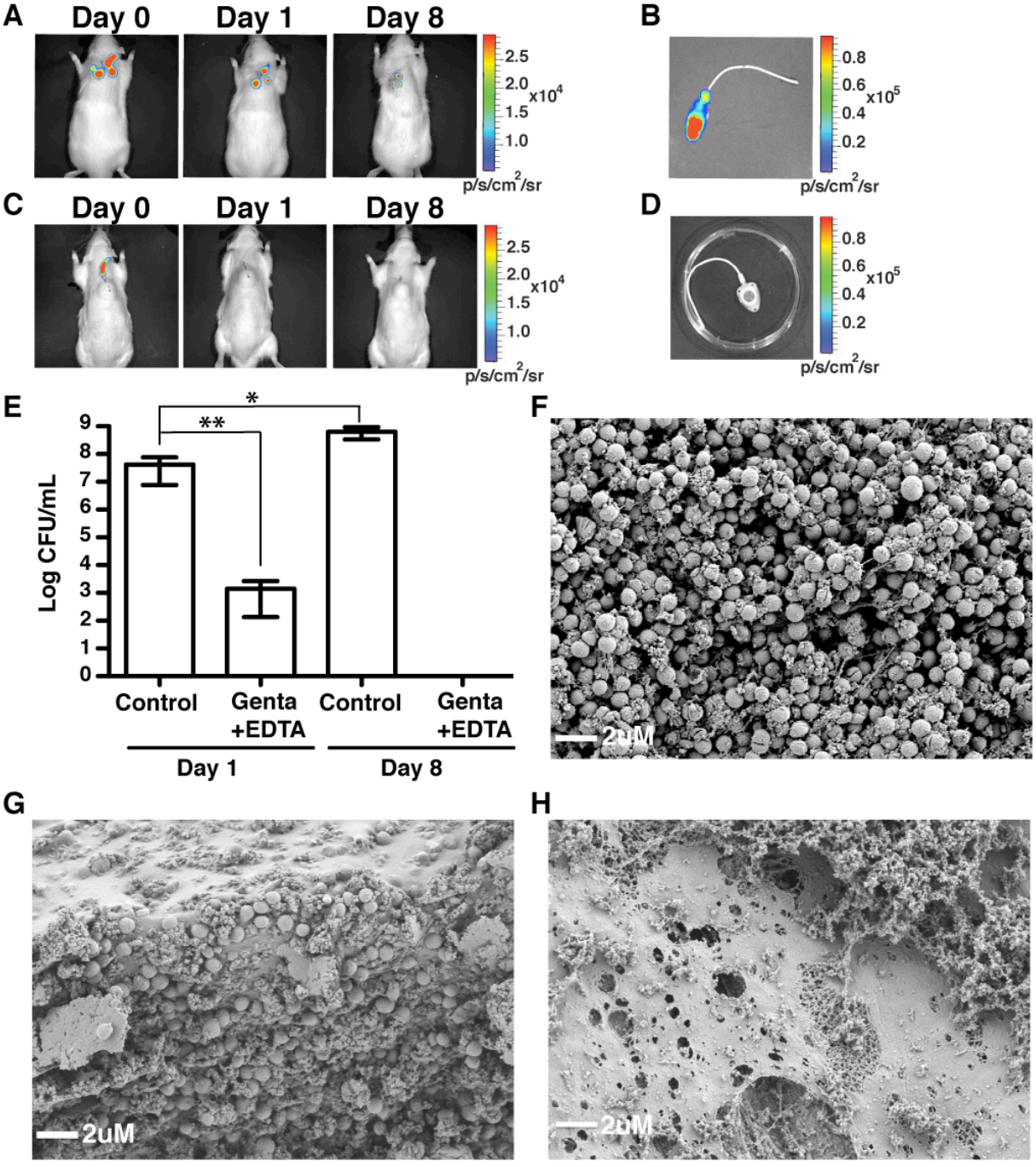


Figure 1. Chauhan *et al.*



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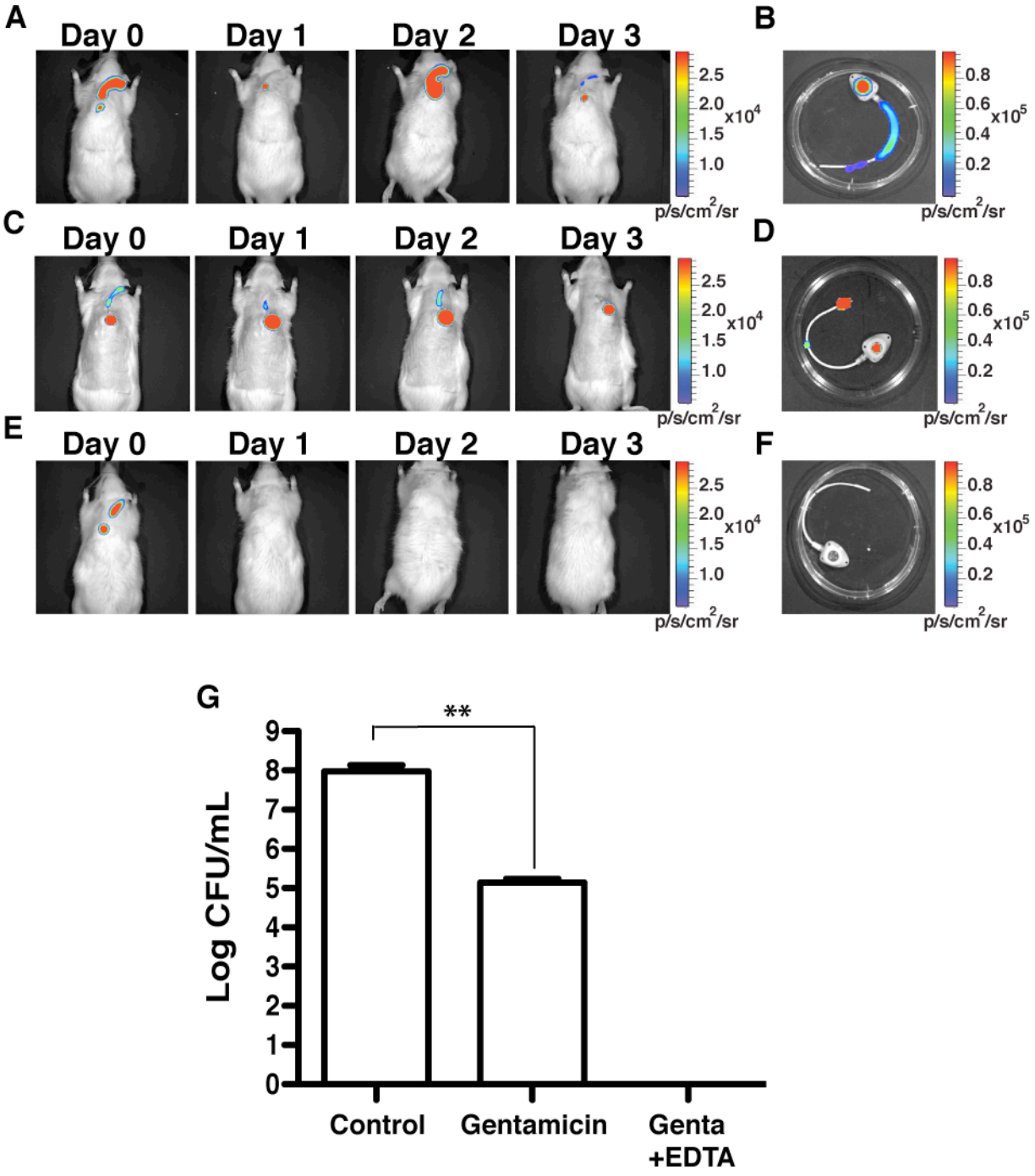

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596 **Figure 3.** Chauhan *et al.*

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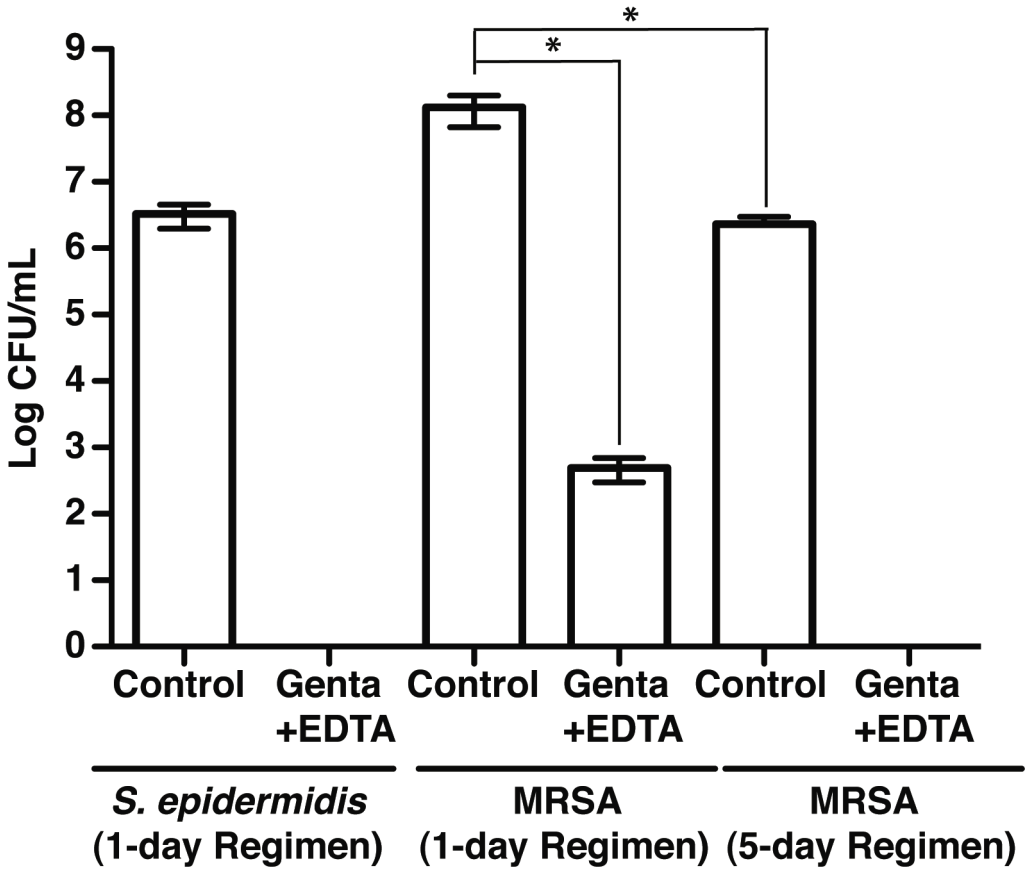

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601 **Figure 4.** Chauhan *et al.*

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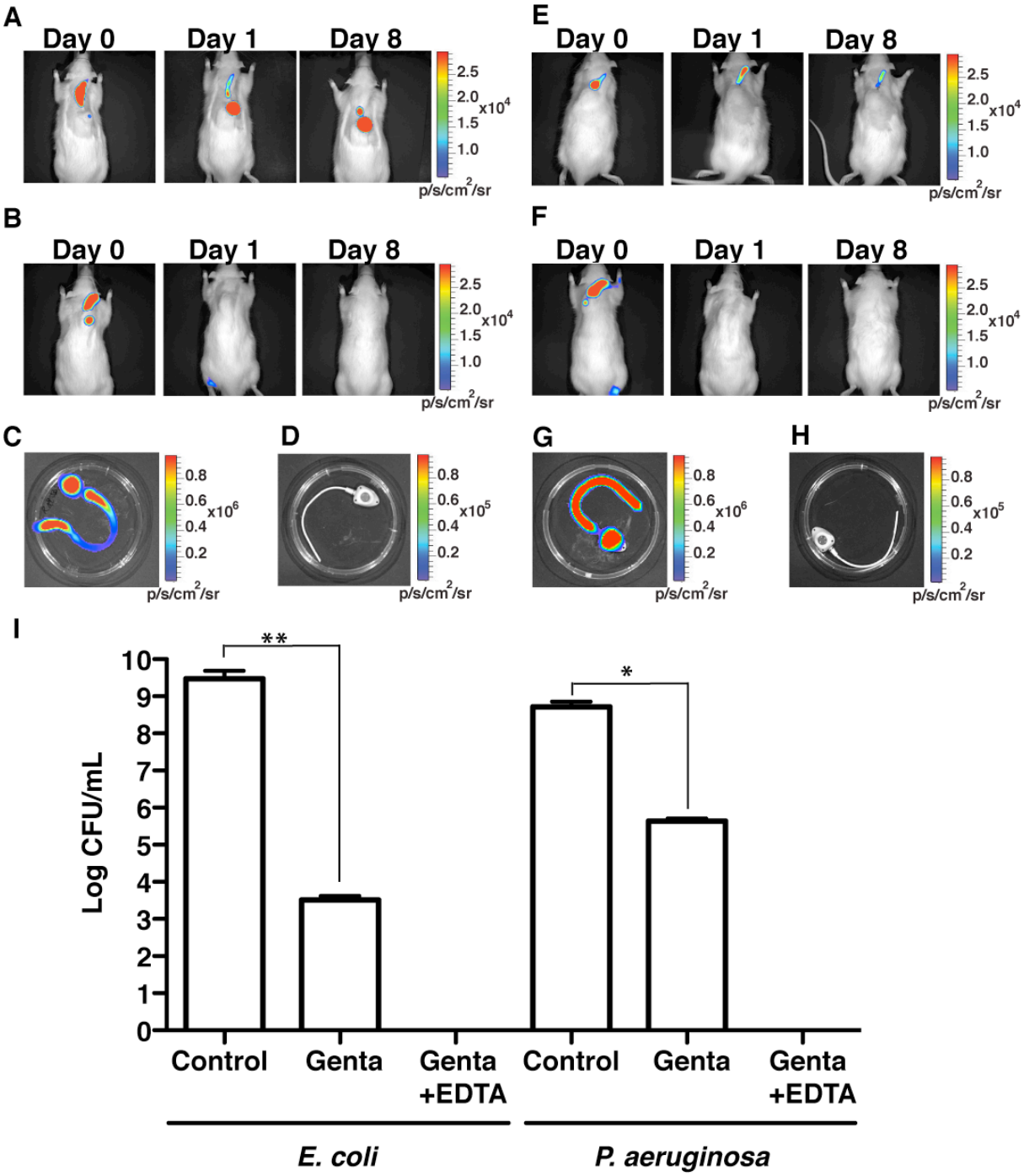

Figure 5. Chauhan *et al.*