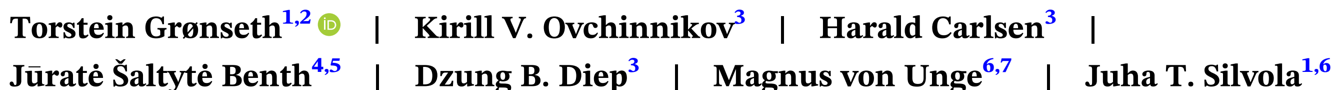
Received: 6 February 2022 | Revised: 6 May 2022 | Accepted: 7 May 2022

DOI: 10.1111/iwj.13846

ORIGINAL ARTICLE

IWJ WILEY

Lugol's solution and Gentian violet eradicate methicillin-resistant *Staphylococcus aureus* biofilm in skin wound infections



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Funding information

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Abstract

The study aimed to evaluate the antibacterial efficacy of Lugol's solution 5% and Gentian violet 1% against methicillin-resistant *Staphylococcus aureus* (MRSA) biofilm in vivo. The bactericidal efficacy for treatment of MRSA-biofilm skin wound infection was tested in a murine model. Luciferase-tagged *S. aureus* Xen31, a MRSA-strain derived from *S. aureus* ATCC-3359130, was used for infection. Wounds were made in the skin of mice and infected with MRSA. The mice were treated with Lugol's solution and Gentian violet. Application of the antimicrobial agents started 24 hours post infection and was repeated daily for five-days. The antimicrobial effect on the biofilm bacteria was evaluated by measuring bioluminescence from MRSA daily for seven-days. Lugol's solution and Gentian violet showed a significant reduction in luminescent signals from the first assessment day to all subsequent days ($P < .001$). Lugol's solution and Gentian violet effectively eradicated MRSA in biofilm in vivo and could be alternatives or in addition to topical antibiotics when MRSA-biofilm wound infection is suspected.

KEYWORDS

biofilm, gentian violet, Lugol's solution, MRSA, *Staphylococcus aureus*

Key Messages

- *Staphylococcus aureus* commonly form biofilms in skin wound infections, and may be difficult to treat with traditional antibiotics. Eradication of chronic biofilm infection is of importance for successful wound healing. Furthermore, MRSA infections are a rising challenge in many counties
- this study aims to evaluate the effect of two different inexpensive non-prescription antiseptics, Lugol's solution and Gentian violet, on MRSA biofilm in a murine skin wound infection model
- Lugol's solution and Gentian violet effectively eradicated MRSA in biofilm in vivo and could be used as alternatives or in addition to topical antibiotics when MRSA-biofilm wound infection is suspected. Furthermore, the use of

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antiseptics can reduce the overuse of antibiotics to prevent the increasing antibiotic resistance around the world

1 | INTRODUCTION

Multidrug resistant bacteria causing skin, wound, soft tissue infections, sinusitis and otitis media chronica, are a major public health problem.[1] One bacterium commonly encountered in chronic suppurating wounds is *Staphylococcus aureus* (*S. aureus*), which has become resistant to many antibiotics, and includes the notorious Methicillin-resistant *S. aureus* (MRSA).[2] In addition, *S. aureus* is able to produce biofilm, resulting in an even less effective response to antibiotics.[3-7] The need for extended treatment alternatives is obvious.

Although not a new treatment, antiseptics could be one option. In 1867 Joseph Lister published a paper in The Lancet on the application of antiseptics, which paved the way for antiseptic surgery. It saved thousands of patients from lethal infections acquired during and after surgery.[8] However, the use of antiseptics fell from favour due to concerns about antiseptic toxicity such as that from Lister's carbolic wound spray and antiseptics containing mercury- or arsenic-based compounds.[9] Another contributing factor was the discovery of antibiotics.[10] The development of multidrug-resistant bacteria has increased interest in antiseptics as an alternative or additive to antibiotics. Antiseptics have several advantages over antibiotics, such as the capability to target several sites in the microorganism instead of one specific site, and hence with less risk of antimicrobial resistance development, and a broader spectrum of antimicrobial activity against different groups of bacteria, fungi, viruses and protozoa.[11,12]

Lugol's solution and Gentian violet have been used as antiseptics in medical practice since the 19th century. In 1829 the French physician J.G.A. Lugol created the disinfectant Lugol's solution, consisting of 5 g iodine (I2) and 10 g potassium iodide (KI) mixed with 85 mL distilled water.[13] The antimicrobial effect is caused by free iodine penetrating the cell wall, by oxidation and by substitution of microbial content with free iodine.[14] Gentian violet solution is attributed to the French chemist Charles Lauth who, in 1861, synthesised it under the name of "Violet de Paris". In 1891 it was introduced as an antiseptic by Stilling, and marketed as Pyoctanin.[15] It is active against *S. aureus* colonised atopic eczema, reducing both bacterial load and severity of the eczema.[16] The exact mode of the antibacterial mechanism of Gentian violet is not completely known or described. However, suggested modes of action are interference with the protein synthesis, bacterial wall, metabolic processes and by redox reaction.[15]

Previous studies have shown that Lugol's solution and Gentian violet are effective against bacteria in biofilms and planktonic form in vitro but there is little evidence of the effect on MRSA living in biofilm in vivo.[17,18] This study aims to evaluate the effect of Lugol's solution and Gentian violet on MRSA biofilm in a skin wound infection in vivo.

2 | MATERIAL AND METHODS

2.1 | Bacterial strain

S. aureus Xen31 (Perkin Elmer, Waltham, MA, USA), a known biofilm producer, was used for wound inoculation. It is an MRSA strain derived from *S. aureus* ATCC 33591, clinically isolated from Elmhurst Hospital in New York. It is luciferase-tagged, and possesses a stable copy of the modified *Photorhabdus luminescens luxABCDE* operon at a single integration site on the bacterial chromosome.[19,20]

2.2 | Bacterial suspension preparation

S. aureus Xen31, which was stored at −80°C, was transferred onto Mueller-Hinton agar plates and incubated for 18–24 hours at 37°C ± 1°C. Single colonies were then transferred into Brain Heart Infusion (BHI) broth (Oxoid, United Kingdom). The bacterial suspension was incubated aerobically at 37°C ± 1°C without shaking before use for further testing.

2.3 | Test substance

The study evaluated the antimicrobial efficacy of Gentian violet 1% (Oslo University Hospital, Oslo, Norway), Lugol's solution 1% and Lugol's solution 5% (Oslo University Hospital, Oslo, Norway). The active substance of all antiseptics used were pre-dissolved in sterile H_2O to get the desired concentrations. Concentrations were selected based on our broth dilution test and on previous studies.[17,18]

Fucidin 2% (fusidic acid) cream (LEO Pharma A/S, Ballerup, Denmark) was used as a positive control.[21] For the negative control group, no treatment or substance was added to the wounds during the experiment.

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2.4 | MBC of antiseptics against planktonic MRSA

A modified broth dilution test on 96-well microtiter plates was employed to evaluate the efficacy of Gentian violet 1% and Lugol's solution 1% on the planktonic growth of bacterial strain.[22] Microtiter plates (Nunclon Delta Surface, Thermo Fisher Scientific, Roskilde, Denmark) were prepared for serial dilution as follows: 100 μL of the antiseptic solution was serially diluted in BHI, in a two-fold manner, from well column 1 to 10. For growth control (without any added inhibitory substance) 100 μL of BHI broth was transferred by pipette into well column 11, and for sterility control 100 μL of BHI was transferred to well column 12. To each well of columns 1–11, a volume of 100 μL of the bacterial suspension was added to achieve the inoculum of 5×10^5 cfu/mL. The microtiter plate was incubated at 37°C ± 1°C for 16–20 hours before transferring 10 μL from each well to Mueller-Hinton agar plates for verification of the breakpoints. Growth or no growth was evaluated after 18 hours of incubation at 37°C ± 1°C. MBC was defined as the lowest concentration of the antimicrobial agent that inhibited the growth of the tested isolate. The experiment was repeated three times.

2.5 | Disc diffusion susceptibility test

To verify the sensitivity of *S. aureus* Xen31 to the Fucidin 2% cream, diffusion test according to the EUCAST disk diffusion method, version 5, was used. First, the bacterial strain was plated on blood agar plates and incubated for 18 hours at 37°C ± 1°C. Single colonies from blood agar plates were collected and transferred into sterile saline. The suspension was measured to McFarland 0.5 and then spread on Mueller-Hinton agar plates using an automated plate spreader. A 10 μg fusidic acid antimicrobial susceptibility discs (Oxoid, Thermo Scientific) was applied to the agar plates. Inhibition zones were evaluated after 18 hours of incubation at 37°C ± 1°C. The experiment was repeated three times.

2.6 | Murine experiment

2.6.1 | Mice

Four-week-old female BALB/cJRj mice (n = 48) were purchased from Janvier (Le Genest-Saint-Isle, France) through Naiser, Norway. Four to five mice per cage were housed in individually ventilated disposable cages (Innovive, San Diego, CA, USA) during the whole experiment at the laboratory animal facility laboratory at the Norwegian University of Life Sciences, Norway. The housing facilities had a humidity of 45% to 55% and temperature between 23°C and 25°C and with a 12 hours light/dark cycle. The mice were given a regular chow diet (RM1; SDS Diet, Essex, United Kingdom) and water ad libitum. The mice were acclimatised to the facilities for 2 weeks prior to the experiment. At the end of each experiment the mice were anaesthetised by 3% inhalation vaporised Isoflurane (described under "Application of antimicrobials and imaging") and euthanized by cervical dislocation. Of the 48 mice enrolled into the study two mice died during Day 1 after the first but before the second treatment. One mouse was in the Lugol's solution 1% group and one in the Lugol's solution 5% group. On Day 4, a mouse in the negative control group partly lost its Tegaderm film (3 M Medical Products, St. Paul, MN, USA) and was therefore excluded from that day on since this could have affected the measurements.

2.6.2 | Wound preparation and inoculation of MRSA

Mice were anaesthetised with a ZRF cocktail before wound preparation and the inoculation of MRSA. The ZRF cocktail consists of 3.3 mg Zoletil Forte (Virbac, Carros, France), 0.5 mg Rompun (Bayer, Oslo, Norway), and 2.6 μg Fentadon (Eurovet Animal Health, Bladel, The Netherlands) per ml 0.9% NaCl. ZRF was administered by intraperitoneal injection at 0.1 mL ZRF/10 g body weight.

After anaesthetising the mice, fur on the back and flanks were removed first with an electrical hair trimmer (Wella Professional Contura HS 61 Trimmer). The remaining fur was removed with a hair removal cream (Veet, Reckitt Benckiser, Slough, United Kingdom) to secure Tegaderm adhesion. One wound was made in the skin on the back of each mouse with a 6 mm sterile disposable biopsy punch (Miltex Instruments, Bethpage, NY, USA). Each wound was inoculated with 20 μL of ice-cold phosphate-buffered saline (PBS) containing 6×10^7 CFUs of *S. aureus* Xen31 using a pipette with tip. After bacterial application, the mice were kept on a warm pad for 10–15 min to dry the inoculum. Wounds of all the mice from all groups were then covered with a 4 × 5 cm Tegaderm film to prevent contractures of the skin and to keep the wound from later drying out and crusting. The Tegaderm also keeps the mice form licking and cleaning the wound. The mice were then placed back in cages for 24 hours to establish the wound infection.

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2.6.3 | Application of antimicrobials and imaging

Twenty-four hours post infection the mice were first subjected to in vivo bioluminescent imaging followed by application of defined formulations according to experimental groups (three different treatments, positive control and negative control). One advantage of the bioluminescent imaging method is that each mouse can be evaluated repeatedly, and thus reducing the number of mice needed. Twenty-four hours post infection was referred to as "Day 1". Imaging and antimicrobial application were repeated every 24 hours for 5 days. On the last 2 days of the experiment, only imaging was performed.

First the mice were anaesthetised by 2.0% vaporised Isoflurane (IsoFlo vet., Zoetis, Zaventem, Belgium), administered with room air at a flow rate of 0.5–1.0 L/min. Anaesthetised mice were next placed in a light tight imaging chamber and kept immobilised by Isoflurane. Photons from luciferase tagged MRSA were measured from mouse wounds over a one-minute period (IVIS Lumina II, Perkin Elmer, Waltham, MA, USA) and light intensity expressed as photons per second per square centimetre per steradian (p/s/cm^2/sr) using Living Image software (Perkin Elmer). Light intensity is proportional to the amount of live bacteria and gives a quantitative result.[23]

Immediately after imaging, an insulin syringe (BD SafetyGlide; 29G needle) was used to inject 50 μL of the antibacterial agent into the wound area underneath the Tegaderm. Mice in the negative control group were left untreated over the entire course of the experiment (Day 7).

2.6.4 | Scanning electron microscope of biofilm

S. aureus Xen31 is a known biofilm producer in vitro.[24] To verify that the strain also produced biofilm in the mouse wounds, and that we were treating bacteria living in biofilm, a scanning electron microscope (SEM) experiment was set up using a selection of mice (n = 4). The procedure for wound infection was similar to the murine experiment described above, except for one mouse serving as negative control (not infected). Twenty-four hours post infection mice were imaged for a luminescent signal from the wounds as described above. The mice were then anaesthetised with the same ZRF cocktail as used before and the skin around the wound area with the Tegaderm film was sampled for further SEM analysis. After the samples were taken each mouse was euthanized by cervical dislocation.

Biopsy samples were first carefully washed twice in PBS before being fixated in a stepwise manner in 50 mL 2% paraformaldehyde, 25 mL 4 M PBS, 5 mL 1.25% glutaraldehyde and 20 mL distilled H_2O. Subsequently, samples were dehydrated in increasing alcohol series of 30%, 50%, 70%, 90%, 96% ethanol for 10 min each, followed by 4 × 10 min in 100% ethanol. The samples were then subjected to critical point drying and sputter-coated with a palladium–gold thin film before examination at 12 kV by SEM (Zeiss EVO 50 EP scanning electron microscope, Carl Zeiss AG, Germany).

2.7 | Statistical analysis

Luminescent signals were presented as means and SDs within the groups at each time point. For further analyses, luminescent signals were (natural) log-transformed, as the distribution was skewed. Due to repeated measurements for each mouse, within-mouse correlations were likely to be present. Therefore, a linear mixed model with random intercepts for mice was estimated to assess the differences in trend in luminescent signal between the groups. The model contained fixed effects for time (third-order polynomial), group (negative controls as reference) and the interaction between them. A significant interaction implies that there are overall differences between the groups in trend in luminescent signal. Post hoc pair-wise comparisons were performed to assess differences between the negative control group and other groups at each time point as well as changes within the groups. A standard residual diagnostic was performed. One author, JSB, is professional biostatistician and was responsible for statistical analyses.

Statistical analyses were performed using SPSS statistical software (release 25.0 SPSS Inc., Chicago, Il, USA) and SAS EG 7.1. Results with $P < .05$ were considered statistically significant.

2.8 | ETHICS STATEMENT

This study was carried out in accordance with the 3Rs (Replacement, Reduction, Refinement) principle and ARRIVE guidelines. Experiments were approved by the Norwegian Food Safety Authority (FOTS), with application no. FOTS ID 20834. Mice were scored daily in accordance with a general distress scoring sheet modified from Wolfensohn and Lloyd 2008. None of the mice showed any adverse, predefined harmful wound effects that could have led to humane endpoints of the study. Small wounds are known to be well tolerated by mice without the need for continuous pain killer treatment.

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3 | RESULTS

3.1 | MBC of antiseptics against planktonic MRSA

Using *S. aureus* Xen31 as indicator, Gentian violet in the broth dilution test had an MBC of 0.3 μg/mL. For Lugol's solution the MBC was 375 μg/mL. This indicates that both are effective antimicrobials against *S. aureus* Xen31 in planktonic form in vitro.

3.2 | Disc diffusion susceptibility test

In the diffusion susceptibility test showed *S. aureus* Xen31 to be sensitive to fusidic acid. The inhibition zones were measured to 28 mm.

3.3 | Murine experiment

All groups had pus filed wounds covered by Tegaderm and showed strong bioluminescent signals indicating viable bacteria in wounds before the first administration of the active formulation (Figure 1). There was a clear non-linear trend in log-transformed luminescent signal values in all groups (Figure 2). Significant interaction terms imply that the overall trend was significantly different between the negative control group and all other groups except for the group "Lugol's solution 1%".

For Lugol's solution 5% the change of emission of photons from Day 1 was significant to all subsequent days (all $P < .001$). The differences between Lugol's solution 5% and the negative control group at each day were significant from 24 hours after the first administration until Day 7 (all $P < .001$) (Table 1). There were no increase in bioluminescent signal intensity indicating no surviving viable bacterial cells on Day 7, even though the application was terminated on Day 5.

For Lugol's solution 1% there was significant emission of photons from the wounds from 24 hours after the first application of the antiseptic until termination on Day 4 (Figure 1). The group was terminated on Day 4 since there was strong emission of photons from the infected wounds, indicating a non-successful eradication the bacteria in the biofilms. The change in emission of photons was only significant from Day 1 to Day 4 ($P = .027$). Comparison of the photon emission of Lugol's solution 1% to negative control at each day, revealed significantly higher values in Lugol's solution 1% group on Day 2 and Day 3 while no significant difference was noted on Day 4 (Table 1).

For Gentian violet 1% the change in emission of photons from Day 1 to all subsequent days was statistically significant (all $P < .001$). Comparing Gentian violet 1% to negative control showed significantly lower values in Gentian violet 1% group at each day from 24 hours after

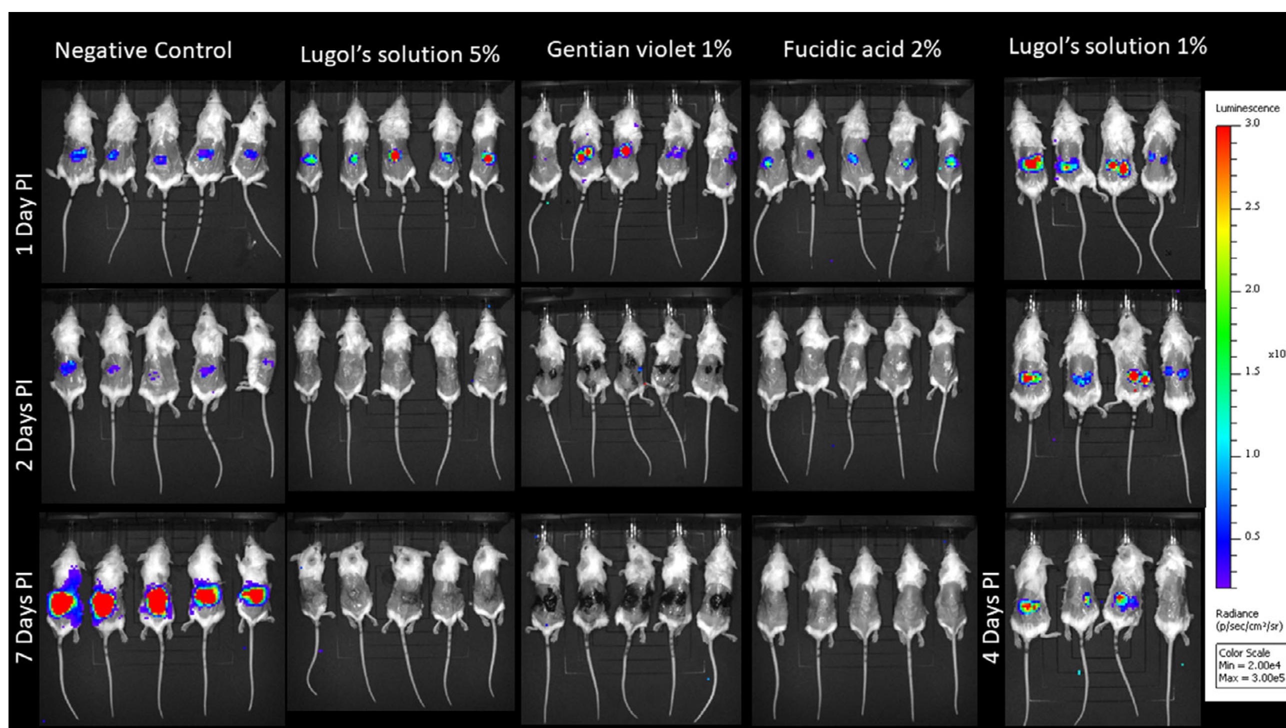

FIGURE 1 Luminescent signals were quantified as photons per second per square centimetre per steradian (p/s/cm^2/sr)

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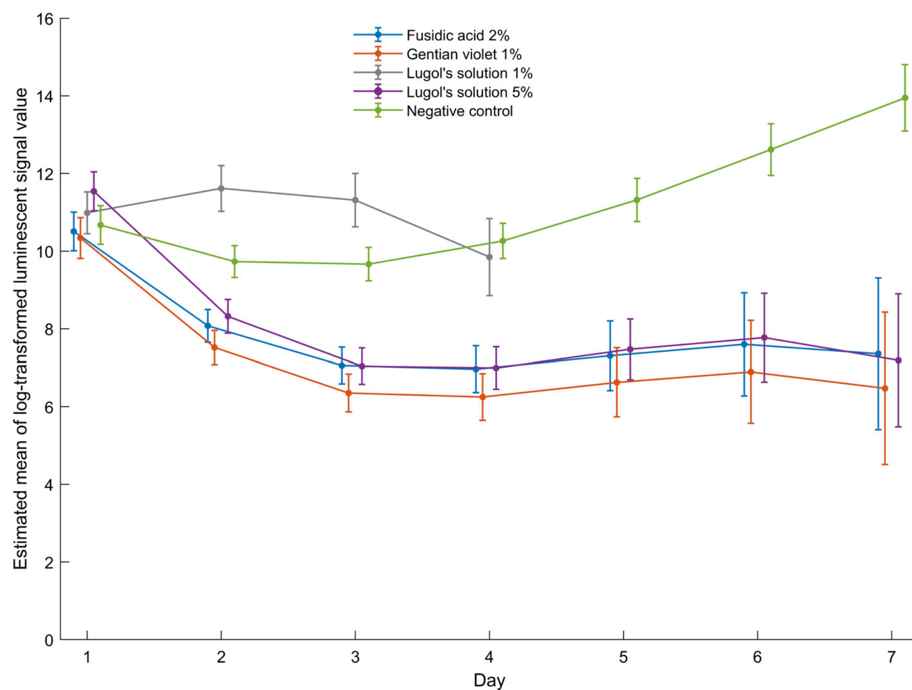

FIGURE 2 Estimated mean values of log-transformed luminescent signal with 95% confidence interval presented as error bars

the first administration until termination at Day 7 (all $P < .001$) (Table 1). There were no increase in bioluminescent signal intensity indicating no surviving viable bacterial cells on Day 7, even though the application was terminated on Day 5. This is visible from the photos showing the reduced emissions compared with the negative control (Figure 1).

S. aureus Xen31 is sensitive to fusidic acid in vitro and therefore Fucidin was used as a positive control.[21] As expected, the positive control group showed a significantly lower emission of photons compared to the negative control from 24 hours after first application until termination (all $P < .001$). Furthermore, the change from Day 1 to Days 2, 3, 4, 5, 6 (all $P < .001$) and Day 7 ($P = .002$) was statistically significant (Figure 2, Table 1).

The mice in the negative control group left untreated showed a strong signal throughout the experiment which got stronger towards termination (Figure 1).

The mice displayed no need for additional pain medication. None of the mice lost more than 10% of body weight, and none seemed distressed by their wounds, with no scratching. The wounds remained open during the whole experiment for all groups due to the Tegaderm. Neither edema, elevated borders nor rubor were observed around the wounds.

3.3.1 | Scanning electron microscope of biofilm

Bioluminescent imaging of wounds showed clear signals from the wounds of the three infected mice 24 hours post infection, while the negative control mouse (non-infected) had no signal (Figure 3). This indicates that *S. aureus* Xen31 were viable in the wounds before the biopsy for SEM. Analysis of the wound samples by SEM showed that the wound edges of infected mice showed typical morphology of biofilm architecture of multilayered aggregates of bacterial cells with interspersed extracellular polymeric substance surrounding the cell clusters (Figure 3).[25] This shows that biofilm was present in the wound 24 hours post infection, before the first treatment dose was applied.

4 | DISCUSSION

Lugol's solution 5% and Gentian violet 1% showed a significant antimicrobial effect against MRSA (*S. aureus* Xen31) in biofilms. We took several measures to strengthen the evidence. First, we used a murine model that involves the production of large quantities of pus in the wounds. This is important because antiseptic formulations can be sensitive to organic material and to changes, such as pH, in the environment.[14,26] The pus can also dilute the active agent thereby reducing its potency. Second, the time from inoculation of the wounds until the first application of the antimicrobial agent was 24 hours to allow sufficient time for creating an established and robust biofilm infection, which is common in clinical settings.[27-31] Third, the application of antimicrobials was stopped on Day 5, 48 hours before the last measurement of the luminescent signals. This ensured that any

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TABLE 1 Antiseptic effects vs control

	Negative control Statistic	Fucidin 2% cream Statistic	*P*-value	Gentian violet 1% Statistic	*P*-value	Lugol's solution 1% Statistic	*P*-value	Lugol's solution 5% Statistic	*P*-value
Day 1									
N	10	10		9		9		10	
Mean (SD)	43 843 (18307)	51 247 (29319)	0.645	111 399 (155624)	0.365	105 736 (104582)	0.401	196 037 (199782)	**.016**
Day 2									
N	10	10		9		8		9	
Mean (SD)	19 551 (9330)	2370 (851)	**<0.001**	707 (253)	**<0.001**	157 464 (144079)	**<0.001**	1830 (367)	**<.001**
Day 3									
N	10	10		9		8		9	
Mean (SD)	19 624 (6322)	1351 (334)	**<0.001**	1019 (235)	**<0.001**	175 167 (226309)	**<0.001**	1590 (376)	**<.001**
Day 4									
N	9	10		9		8		9	
Mean (SD)	23 840 (12770)	1459 (400)	**<0.001**	824 (282)	**<0.001**	66 036 (87539)	0.402	1604 (651)	**<.001**
Day 5									
N	9	10		9				9	
Mean (SD)	148 046 (158530)	1421 (447)	**<0.001**	839 (447)	**<0.001**			1537 (442)	**<.001**
Day 6									
N	9	10		9				9	
Mean (SD)	670 801 (621234)	1688 (372)	**<0.001**	581 (105)	**<0.001**			1550 (206)	**<.001**
Day 7									
N	9	10		9				9	
Mean (SD)	1 109 078 (474201)	1809 (468)	**<0.001**	879 (316)	**<0.001**			1573 (540)	**<.001**

Note: A comparison of antiseptic effects vs control, descriptive numbers. Mean luminescent signal is quantified as photons per second per square centimetre per steradian ($p/s/cm^2/sr$). *P*-values follow from post hoc analysis of linear mixed model presented in Table 2. Bold values indicate $P < .05$.

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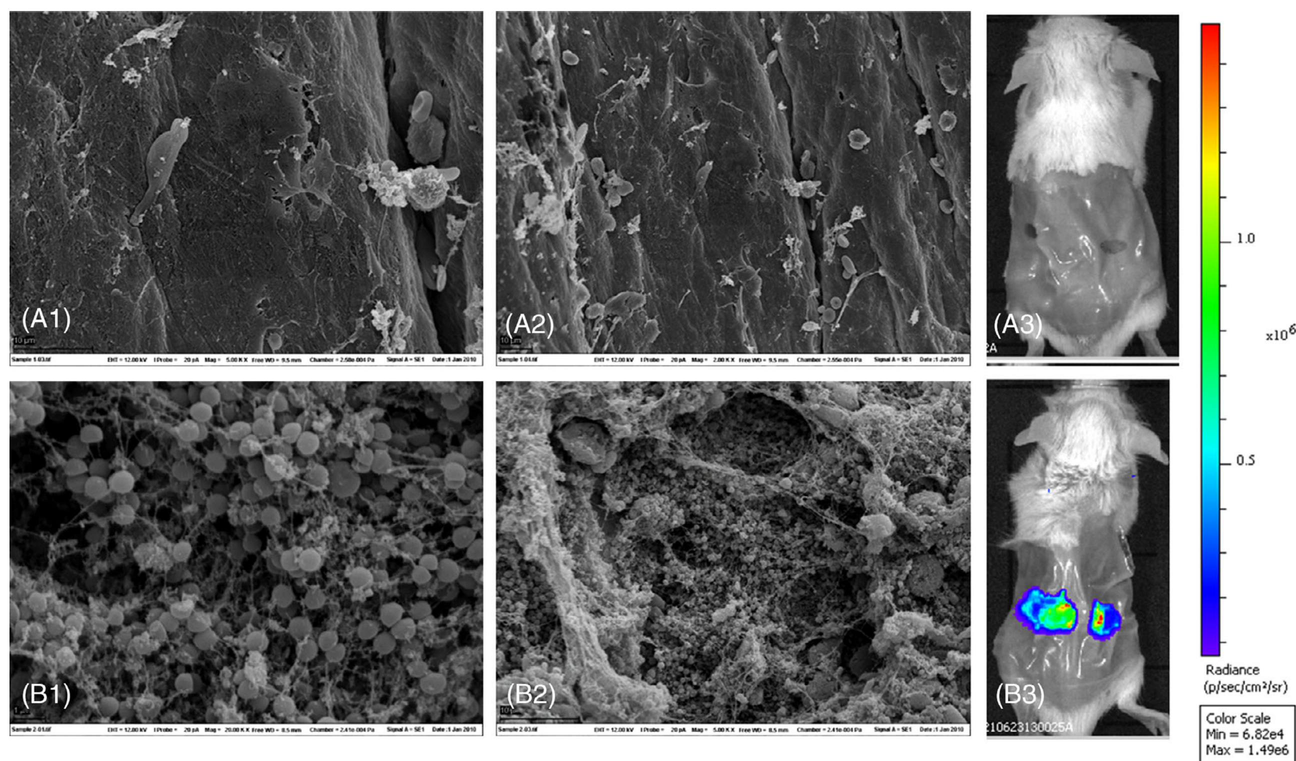

FIGURE 3 Negative control: (A1-A2) scanning electron microscope (SEM) of wound edge. (A3) image of luminescence (p/s/cm^2/sr). Infected mice with MRSA: (B1-B2) SEM of wound edges showing biofilm with interspersed extracellular polymeric substance surrounding the cell clusters. (B3) image of luminescence (p/s/cm^2/sr). SEM scale bar on each image. Scale bar for image of luminescent signal right (p/s/cm^2/sr)

dormant or surviving bacteria would have time to replicate and thus start to emit luminance. Fourth, we used Tegaderm to cover the wound area, to prevent it from contracting, which is the main route to healing on loose-skinned animals like mice. In addition, Tegaderm prevents the mice from licking off the applied antimicrobial substance over the wound. In this way, our skin infection model more resembles human skin wound infection which heals primarily by re-epithelization.[32,33] This wound model with a cover of Tegaderm can mimic a closed cavity infection, like an abscess, otitis media or sinusitis.

Although this study shows promising results for treating *S. aureus* living in biofilm in vivo, more studies need to be conducted to make further conclusions. First, this is a single laboratory bacterial strain study, and studies have shown that there may be a difference in tolerance between clinical strains and laboratory strains.[18,34] Second, in clinical wound infections, the biofilm is often older than 24 hours and consists of different species, both Gram negative and Gram positive, which may increase antimicrobial tolerance.[35] Replicating this study with different bacterial strains and with different ages of the biofilm would be needed.

4.1 | Lugol's solution

We have not found previous studies describing the effect of Lugol's solution on MRSA in vivo. We evaluated Lugol's solution at both 1% and 5%. Lugol's solution 5% showed a significant reduction in luminescent signals from 24 hours after the first application until termination. There was clearly no luminescent signal on the scans. Even 48 hours after the last application (Day 7), the signal had not picked up any indication of surviving bacteria. This suggests that Lugol's solution 5% can be an effective formulation and concentration in treating infections of MRSA living in biofilms.

Our previously published study showed that Lugol's solution 1% was effective against *S. aureus*, both in the planktonic and the more resilient biofilm form in vitro.[17] Based on these results, one could expect the 1% solution to be effective in vivo. Nonetheless, in this study we found no significant reduction in luminescent signals in mice treated with Lugol's solution 1%, which clearly indicated surviving bacteria. The lack of effect of Lugol's solution 1% could be due to the dilution caused by pus and exudate in the wound, or possibly by the neutralisation of free iodine by organic compounds in the wound.[26]

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TABLE 2 Results of linear mixed model

Parameter	Regression coefficient (SE)	*P*-value
Intercept	12.70 (0.64)	<.001
Day	−2.64 (0.63)	<.001
Day^2	0.65 (0.18)	<.001
Day^3	−0.04 (0.01)	.020
Group		
Fusidic acid 2%	2.14 (0.90)	.019
Gentian violet 1%	2.69 (0.93)	.004
Lugol's solution 1%	−3.03 (2.07)	.144
Lugol's solution 5%	4.71 (0.92)	<.001
Negative control – ref.	0	
Day × Group		
Day × Fusidic acid 2%	−2.80 (0.89)	.002
Day × Gentian violet 1%	−3.73 (0.91)	<.001
Day × Lugol's solution 1%	4.23 (3.08)	.171
Day × Lugol's solution 5%	−4.78 (0.91)	<.001
Day × Negative control – ref.	0	
Day^2 × Group		
Day^2 × Fusidic acid 2%	0.54 (0.25)	.031
Day^2 × Gentian violet 1%	0.76 (0.26)	.003
Day^2 × Lugol's solution 1%	−0.88 (1.35)	.515
Day^2 × Lugol's solution 5%	1.03 (0.26)	<.001
Day^2 × Negative control – ref.	0	
Day^3 × Group		
Day^3 × Fusidic acid 2%	−0.05 (0.02)	.028
Day^3 × Gentian violet 1%	−0.06 (0.02)	.004
Day^3 × Lugol's solution 1%	−0.004 (0.18)	.981
Day^3 × Lugol's solution 5%	−0.08 (0.02)	<.001
Day^3 × Negative control – ref.	0	

Note: Results of linear mixed model, natural log-transformed luminescent signal values used as outcome variable.

Furthermore, there was an initial increase in luminescent signals on Day 2 and Day 3, significantly higher than the negative control. One possible explanation is that the concentration of Lugol's solution was too low to kill the *S. aureus* and so, instead of killing the *S. aureus*, it may have caused more exudate, resulting in an initial favourable environment for bacteria growth.

Different formulations containing iodine, like Lugol's solution and different idophores, are commercially available. Their antimicrobial effects are exerted by free iodine.[36] Lugol's solution has a relatively large amount of free iodine. On the other hand, most of the iodine in idophores is bound up in carrier molecules, requiring approximately 2 minutes of contact time to release it. This may be of importance when used in wounds with exudate and a lot of pus that may neutralise the application.[14] In studies of povidone-iodine the results are mixed. In a study comparing the effect of clinical cure rates in patients with soft tissue abscesses, the authors found no differences in the treatment of incising, draining and irrigating, either alone or with the addition of povidone-iodine.[37] In another study only 2% fusidic acid but not 10% povidone-iodine showed significant reduction ($P < .01$) in colony counts of *S. aureus* from biofilm in the croton oil dermatitis mice compared to untreated mice.[38] In a third study, the researchers reported anti-biofilm effect by povidone-iodine on *S. aureus* biofilm on the sinonasal mucous membrane in mice.[39]

We did not find any adverse skin reactions to Lugol's solution in our study. Although we found no studies on possible adverse effects on Lugol's solution, studies performed on povidone-iodine show mixed results in regards to re-epithelization and tensile strength.[40-45] Although possible adverse effects have been described, most clinical studies have concluded that povidone-iodine has no negative effects on wound healing and is safe to use in humans although additional evidence is needed.[46,47]

Fear of an allergic reaction has made many doctors reluctant to use iodine-containing products. Lugol's solution contains only H_2O, potassium iodine and elementary iodine, which are elementary components in the body and therefore fear of allergic reactions should not be of reasonable concern.[48,49]

4.2 | Gentian violet

Even though Gentian violet is a long-established antiseptic, there are few studies assessing its effect on MRSA. What sets our study apart is the use of a biofilm skin wound infection model with no additional confounding treatments. Furthermore, the presence of pus is important because of its dilution effect. Together with exudate, pus causes a change in pH, which can alter the efficacy of Gentian violet.[50] Gentian violet 1% showed a significant reduction in luminescent signals from 24 hours after its first application until termination. Even 48 hours after the last Gentian violet application (Day 7), the signals had not picked up, indicating no surviving bacteria. This rapid bactericidal effect came faster than previously described. In a study of MRSA infected decubitus ulcers, a combination of treatment, consisting of scrubbing the ulcers with Gentian violet aqueous solution 0.1% and a daily application of Gentian violet 0.1% ointment, resulted in eradication of the MRSA within 3–34 days. The eradication time was longer than obtained in our

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study even with the additional effect of mechanical scrubbing.[51] Furthermore, Okano found that Gentian violet 0.5% achieved disinfection of the nasal carriage of MRSA in 15.3 ± 9 days in nine patients.[52] The most plausible reason for faster action in our model is the higher concentration of Gentian violet. Another reason may be the overlying Tegaderm which resulted in the longer and more persistent exposure to Gentian violet.

We did not find any adverse skin reactions to Gentian violet in our study. Other studies have shown Gentian violet to facilitate wound healing in dermatological conditions like pyoderma gangrenosum, epidermolysis bullosa and calciphylaxis, and in foreign body granulomas.[53] When comparing healing time between Gentian violet and other dressings the results are varied, with some showing prolonged healing time while other showing no difference.[54-56]

5 | CONCLUSION

Lugol's solution 5% and Gentian violet 1% have strong antimicrobial effects on MRSA (*S. aureus* Xen31) in biofilms in vivo, as compared to a negative control. Lugol's solution and Gentian violet may have a potential role as an inexpensive treatment option in the treatment of infections with biofilm.

ACKNOWLEDGEMENTS

Special thanks to Hilde Raanaas Kolstad of the Imaging Centre, NMBU, for her help and expertise with the scanning electron microscope used for this study.

CONFLICT OF INTEREST

The authors have no conflict of interest in the content or the materials used in this study.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Grønseth T, Ovchinnikov KV, Carlsen H, et al. Lugol's solution and Gentian violet eradicate methicillin-resistant *Staphylococcus aureus* biofilm in skin wound infections. *Int Wound J*. 2022;1-11. doi:10.1111/iwj.13846

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