Effectiveness of mechanical cleaning, antibiotics, and induction heating on eradication of Staphylococcus aureus in mature biofilms

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Aims

Here we used a mature seven-day biofilm model of *Staphylococcus aureus,* exposed to antibiotics up to an additional seven days, to establish the effectiveness of either mechanical cleaning or antibiotics or non-contact induction heating, and which combinations could eradicate *S. aureus* in mature biofilms.

Methods

Mature biofilms of *S. aureus* (ATCC 29213) were grown on titanium alloy (Ti6Al4V) coupons for seven days and were subjected to the following treatments or their combinations: antibiotics, mechanical cleaning, or heat shock by induction heating of 60°C for one minute. Experiments were repeated at least five times.

Results

In the untreated biofilm, growth up to 1.8×10^{11} colony-forming units (CFU)/cm^2 was observed. Treatment with ciprofloxacin, flucloxacillin, vancomycin, cefuroxime, and amoxicillin all with rifampicin gave 6.0 log, 6.1 log, 1.4 log, 4.8 log, and 3.6 log reduction in CFU/cm^2, respectively. Mechanical cleaning alone resulted in 4.9 log reduction and induction heating in 7.3 log reduction. There was an additional effect of ciprofloxacin, flucloxacillin, and induction heating when used in combinations. There was no additional effect for mechanical cleaning. No bacterial growth could be detected after induction heating followed by seven days of ciprofloxacin with rifampicin.

Conclusion

Mechanical cleaning, antibiotics, and non-contact induction heating reduced the bacterial load of mature *S. aureus* biofilms with approximately 5 log or more as a single treatment. The effect of mechanical cleaning on mature *S. aureus* biofilms was limited when used in combination with antibiotics and/or induction heating.

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Keywords: Implant-associated infection, Staphylococcus aureus, Biofilm, Non-contact induction heating, Antibiotics, Periprosthetic joint infection

Article focus

- Non-contact induction heating (NCIH) of metal implants is a non-antibiotic treatment modality that can potentially be used during debridement, antibiotics, and implant retention (DAIR) to cause thermal damage to the bacterial biofilm, alongside mechanical cleaning and antibiotics.
- We exposed seven-day mature biofilms of *Staphylococcus aureus* to several antibiotics, mechanical cleaning, NCIH, and their combinations.
- We determined the possible synergistic effect of thermal dose by NCIH and seven-day antibiotic exposure at clinically relevant doses.

Key messages

- The combinations of NCIH and antibiotics are key, because neither induction heat,

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antibiotics, nor mechanical cleaning alone were able to achieve full eradication.
- The combination of NCIH and ciprofloxacin with rifampicin was synergistic, and no viable bacteria could be detected after this combination treatment.
- The effect of mechanical cleaning on mature *S. aureus* biofilms was limited when used in combination with antibiotics and/or induction heating.

Strengths and limitations

- We evaluated the effect of treatments that are part of DAIR or can be used during DAIR.
- We conducted multiple experiments on seven-day biofilms of *S. aureus,* which is one of the most commonly isolated pathogens from infected implants.
- Although our results are based on a mature biofilm model seven to 14 days old, these are still in vitro models and may not fully translate to in vivo scenarios.

Introduction

Periprosthetic joint infection (PJI) is a potentially devastating complication following orthopaedic surgery and is often caused by staphylococci such as *Staphylococcus aureus*.[1] These microorganisms form a biofilm on the implant surface, which protects them from the immune system and antibiotics.[1] Additionally, antimicrobial resistance (AMR) is on the rise, which raises concerns and limits the choice of antibiotics.[2,3]

Presently, debridement, antibiotics, and implant retention (DAIR) is considered the treatment of first choice for patients presenting with early PJI.[4-6] The advantage of DAIR is that the well-fixed implant is not removed, resulting in lower morbidity and costs, compared to a one-stage or two-stage revision surgery.[4,6,7] Treatment of PJIs with DAIR is based on mechanical cleaning of the implant surface and subsequent antibiotic therapy. Presently, success rates vary from 60% to 80%,[6,8] which leaves room for improvement. It is therefore paramount that new treatments for PJI are being developed.

Non-contact induction heating (NCIH) of metal implants is a new method that can cause thermal damage to bacterial biofilms, thereby eradicating bacteria and weakening the biofilm. One of the suggested mechanisms by which NCIH could work is disruption of the bacterial membrane by thermal damage, which could lead to bacterial death or bacteria being more susceptible to antibiotics.[9] NCIH only actively heats the metal (implant) and has no direct heating effect on the surrounding tissue; thus, it has been considered as a potential non-invasive treatment for PJI.[10-13] In addition to non-invasive use, NCIH could also be used during surgery of an infected implant to increase the effectiveness of, for example, DAIR.[5,11] NCIH can, for instance, heat parts of the implant that cannot be reached (e.g. posterior femoral condyles), mechanically cleaned, or that are very difficult to clean (e.g. porous coatings). However, the effect of NCIH in a DAIR setting that includes mechanical cleaning and antibiotics is unknown.

Although in vitro biofilm models for PJI can provide valuable insights for the DAIR setting, they are mostly limited to young biofilms of only one day or a couple of days old.[14] Such young biofilms do not represent the mature biofilms present in PJI that are encountered during DAIR, which have typically grown for over a week. Additionally, exposure to antibiotics in young biofilm models is also short, only lasting a few hours, and does not adequately capture the clinical setting of prolonged antibiotic treatment of several weeks. Hence, there is a need for studies using mature biofilm models with exposure to antibiotics that are used in PJI treatment protocols during a clinically relevant period of time. Therefore, we used a mature seven-day biofilm model of *S. aureus,* exposed to antibiotics up to an additional seven days, to answer the following research questions: 1) what is the effectiveness of either mechanical cleaning or antibiotics or non-contact induction heating?; and 2) what combinations can kill *S. aureus* in mature biofilms? We hypothesized that combinations of mechanical cleaning, antibiotics, and non-contact induction heating would be more effective than single treatments.

Methods

Biofilm preparation. Mature biofilms of *S. aureus* ATCC 29213, a biofilm-forming clinical isolate,[14,15] were produced by growth on titanium alloy (Ti6Al4V) coupons (38 mm × 25 mm of 1 mm thickness) for seven days[16] in a polypropylene container equipped with a bacteria filter (1 micron PTFE hydrophobic membrane, Medical Filtration Solutions, UK) to allow for sterile ventilation. This model has been described elsewhere.[17,18] The biofilm was grown by immersing the coupons in 300 ml of growth medium (brain heart infusion (BHI)), inoculated with *S. aureus* (single colony from agar plate) and incubated for seven days at 37°C. The growth medium was not changed during these seven days.

Biofilm treatments. The seven-day biofilms were subjected to the following treatments or their combinations: antibiotics, mechanical cleaning, or heat shock by induction heating. Experiments were repeated at least five times unless otherwise indicated.

Antibiotics. The coupons with seven-day biofilms were gently washed with phosphate-buffered saline (PBS) solution in a petri dish to remove planktonic bacteria. After the PBS wash, the coupons were placed separately into another polypropylene container equipped with a bacteria filter with 50 ml of fresh BHI growth medium that contained one of the following antibiotics: vancomycin (1 mg/l), cefuroxime (10 mg/l), ciprofloxacin (10 mg/l), amoxicillin (25 mg/l), or flucloxacillin (80 mg/l), all from Sigma-Aldrich (USA). The coupons were inoculated for 24 hours at 37°C while exposed to the antibiotic. To mimic clinical practice, we added rifampicin (1 mg/l) to each antibiotic treatment and used a rifampicin-susceptible *S. aureus* strain (ATCC 29213).[4,19] The concentrations for antibiotics were chosen to represent clinically relevant concentrations as they can be expected in the bone.[20] Their minimal inhibitory concentrations (MICs) were 1.5 mg/l for vancomycin, 1 mg/l for cefuroxime, 0.25 mg/l

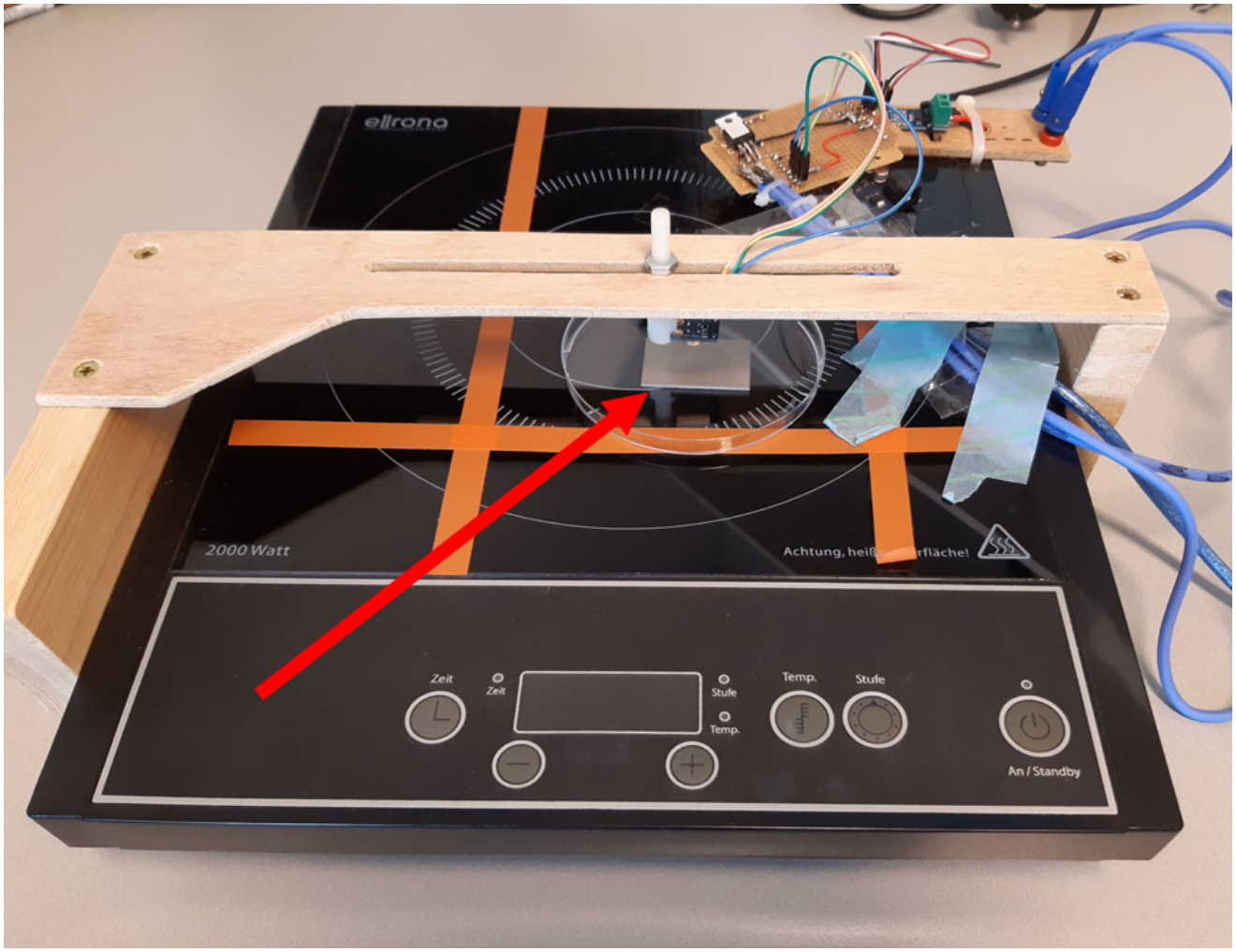

Fig. 1

Photograph of the non-contact induction heating systems and the titanium alloy coupon (Ti6Al4V) in a petri dish. The red arrow shows the Ti6AlV4 coupon and the infrared temperature sensor directly above it for non-contact temperature measurements.

Table I. Details and order of double treatments and triple treatments.

Treatment type	Treatment 1	Treatment 2	Treatment 3
Double treatments	Mechanical cleaning	60°C	-
	Mechanical cleaning	Flu80 Rif1	-
	Mechanical cleaning	Cip10 Rif1	-
	60°C	Flu80 Rif1	-
	60°C	Cip10 Rif1	-
Triple treatments	Mechanical cleaning	60°C	Flu80 Rif1
	Mechanical cleaning	60°C	Cip10 Rif1

60C, induction heating to 60°C for one minute.Cip10Rif1, ciprofloxacin 10 mg/l + rifampicin 1 mg/l; Flu80Rif1, flucloxacillin 80 mg/l + rifampicin 1 mg/l;

for ciprofloxacin, 1 mg/l for amoxicillin, and 0.006 mg/l for rifampicin. After antibiotic exposure, the coupons were washed again with PBS solution in a petri dish, and directly afterward subjected to quantification by direct enumeration.

Mechanical cleaning. Prior to mechanical cleaning, the coupons were gently washed with PBS solution in a petri dish to remove planktonic bacteria. Subsequently, the coupons were exposed to the mechanical cleaning procedure (performed by an orthopaedic surgeon (BGP)), which consisted of rubbing clean the coupon with sterile 10 cm × 10 cm surgical woven gauze swabs for one minute. This was achieved by folding the gauze around the coupon, so that it could be manually cleaned in a sterile way. After mechanical cleaning, the coupons were

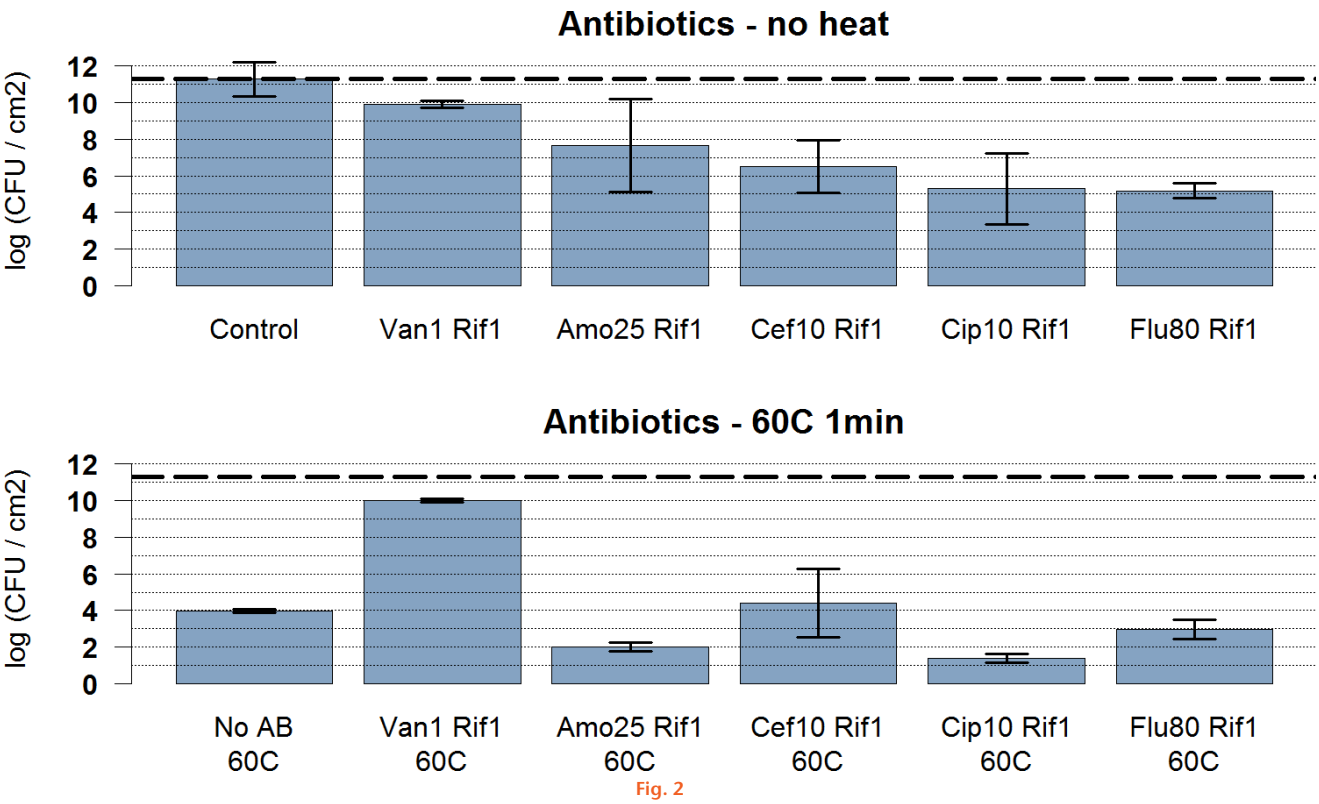

Fig. 2

Seven- to eight-day biofilms: graphs showing the relation between antibiotic exposure and log colony-forming units (CFUs) per cm^2 for seven-day *Staphylococcus aureus* biofilms with (bottom) or without (top) induction heating to 60°C for one minute. The dashed line represents the control. Mean and 95% confidence intervals are presented. No AB, no antibiotics; Van1Rif 1, vancomycin 1 mg/l + rifampicin 1 mg/l; Amo25Rif1, amoxicillin 25 mg/l + rifampicin 1 mg/l; Cef10Rif1, cefuroxime 10 mg/l + rifampicin 1 mg/l; Cip10Rif1, ciprofloxacin 10 mg/l + rifampicin 1 mg/l; Flu80Rif1, flucloxacillin 80 mg/l + rifampicin 1 mg/l; 60C, induction heating to 60°C for one minute.

washed again with PBS solution in a petri dish (similar to the other treatments) and directly afterward subjected to quantification by direct enumeration.

Induction heating. First, the coupons were gently washed with PBS solution in a petri dish to remove any planktonic bacteria, and then they were exposed to a thermal shock of 60°C for one minute. This temperature and duration were chosen as they were expected to be effective and practical while minimizing the risk of necrosis.[13,17,21] The thermal shock was delivered in a non-contact manner using a custom-built induction heater with non-contact infrared temperature control. This system has been validated and described previously (Figure 1).[17,18] In short, the induction heater with a pancake-type coil creates a pulsed electromagnetic field (PEMF) of 97 kHz at a maximum of 65 W to heat the Ti6Al4V coupon in a petri dish that is placed above the pancake coil. The PEMF induces so-called 'eddy currents' in the metal (Ti6Al4V) coupon, which cause it to heat up. For non-contact temperature measurement and temperature control, we used a microcontroller board based on the ATmega328 (Arduino Uno, Adafruit Industries, USA) and infrared temperature sensor (MLX90614; Melexis, Belgium), which has been validated.[17,18] The temperature was recorded four times per second (4 Hz) in real time, and stored in a data file on a laptop. After induction heating, the coupons were washed again with PBS solution in a petri dish (similar to the other treatments) and directly afterward subjected to quantification by direct enumeration.

Combinations of treatment methods. We also subjected the seven-day biofilms to combinations of treatments described above: either double treatments consisting of two treatments or triple treatments consisting of all three treatments (Table I). For the antibiotic treatment, the two most effective antibiotics (as a single treatment) were selected. These were ciprofloxacin (10 mg/l) and flucloxacillin (80 mg/l), both of which were supplemented with rifampicin (1 mg/l) as described above. Prior to and after treatments, the coupons were washed with PBS solution in a petri dish. After treatments, the coupons were subjected to quantification by direct enumeration.

Controls. We also included seven-day biofilm coupons as controls (without treatment), which went through all steps of the aforementioned treatments, including PBS wash, but were not exposed to antibiotics, mechanical cleaning, or induction heating.

14-day biofilm model. In order to simulate the clinical reality of long-term antibiotic treatment, we exposed the seven-day biofilm to seven days of antibiotic treatment with or without induction heating, using the

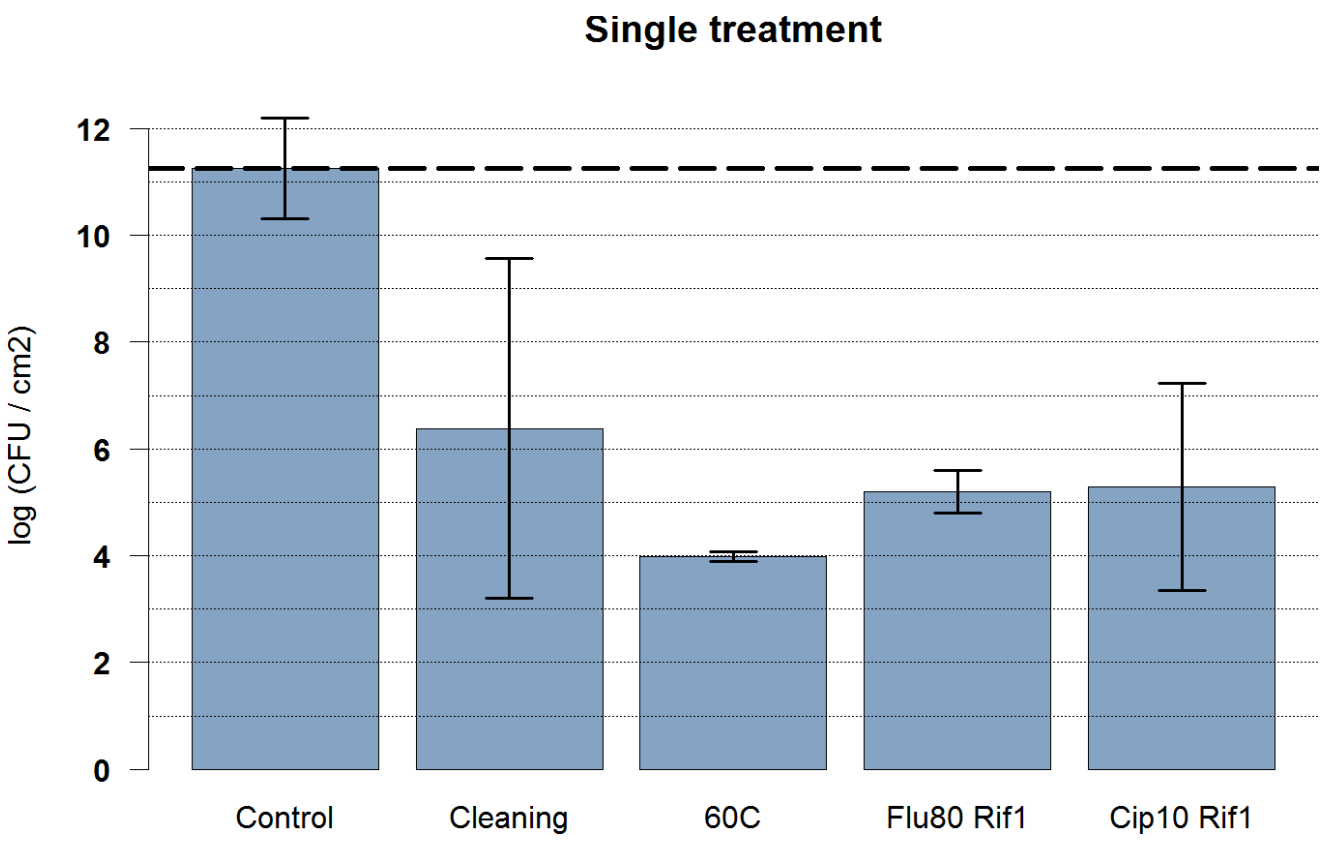

Fig. 3

Seven- to eight-day biofilms: graph showing the log colony-forming units (CFUs) that remain for seven-day *Staphylococcus aureus* biofilms after each single treatment. The dashed line represents the control. Mean and 95% confidence intervals are presented. 'Cleaning' refers to mechanical cleaning. 60C, induction heating to 60°C for one minute; Cip10Rif1, ciprofloxacin 10 mg/l + rifampicin 1 mg/l; Flu80Rif1, flucloxacillin 80 mg/l + rifampicin 1 mg/l.

two most effective antibiotics: ciprofloxacin (10 mg/l) and flucloxacillin (80 mg/l), both supplemented with rifampicin (1 mg/l) as described above. This way, after the treatment any remaining biofilm was 14 days old. Mechanical cleaning was not used in this model, because the random effects model suggested that mechanical cleaning had little to no added value in reducing the bacterial load when used in double or triple combination treatments.

For this biofilm model, the coupons with seven-day biofilm were placed into another polypropylene container equipped with a bacteria filter with 300 ml of fresh BHI growth medium that contained either ciprofloxacin (10 mg/l) and rifampicin (1 mg/l), or flucloxacillin (80 mg/l) and rifampicin (1 mg/l). The coupons were inoculated for an additional seven days at 37°C, and the medium and antibiotics were not replaced during these seven days in order to reduce the risk of contamination. Prior to and after treatments, the coupons were washed with PBS solution in a petri dish. After treatments, the coupons were subjected to quantification by direct enumeration.

Biofilm quantification. After the treatment(s), the coupons were placed in a 50 ml centrifuge tube with 20 ml of PBS solution. This tube, including the coupon, was sonicated (D-78224 Ultrasonic cleaner; Elma Schmidbauer, Germany) for five minutes at 35 kHz to dislodge the bacteria from the biofilm into suspension. Afterwards, a dilution series of the supernatant (centrifuged for five minutes at 12,000 rpm) was cultured for at least 48 hours on BHI plates at 37°C to determine the colony-forming units (CFUs)/cm^2. For the 14-day biofilm model, the dilution series of the supernatant was cultured for a week on BHI plates at 37°C.

Statistical analysis. Statistical analyses, when appropriate, were performed using analysis of variance (ANOVA) (SPSS version 23; IBM, USA). To determine the mean effectiveness of each treatment (e.g. mechanical cleaning) in each combination of treatments, we used a random effects model from the metaphor package (R Foundation for Statistical Computing, Austria).[22] In line with recent recommendations, means and corresponding confidence intervals (CIs) are reported, whereas p-values are not reported.[23] Synergy between two treatments was defined as > 2 log decrease in CFUs/cm^2 between the combination and its most active constituent.[24]

Results

Single treatments. In the control group, 1.8×10^{11} CFU/cm^2 were observed (n = 17). These mature biofilms were macroscopically visible on the titanium alloy

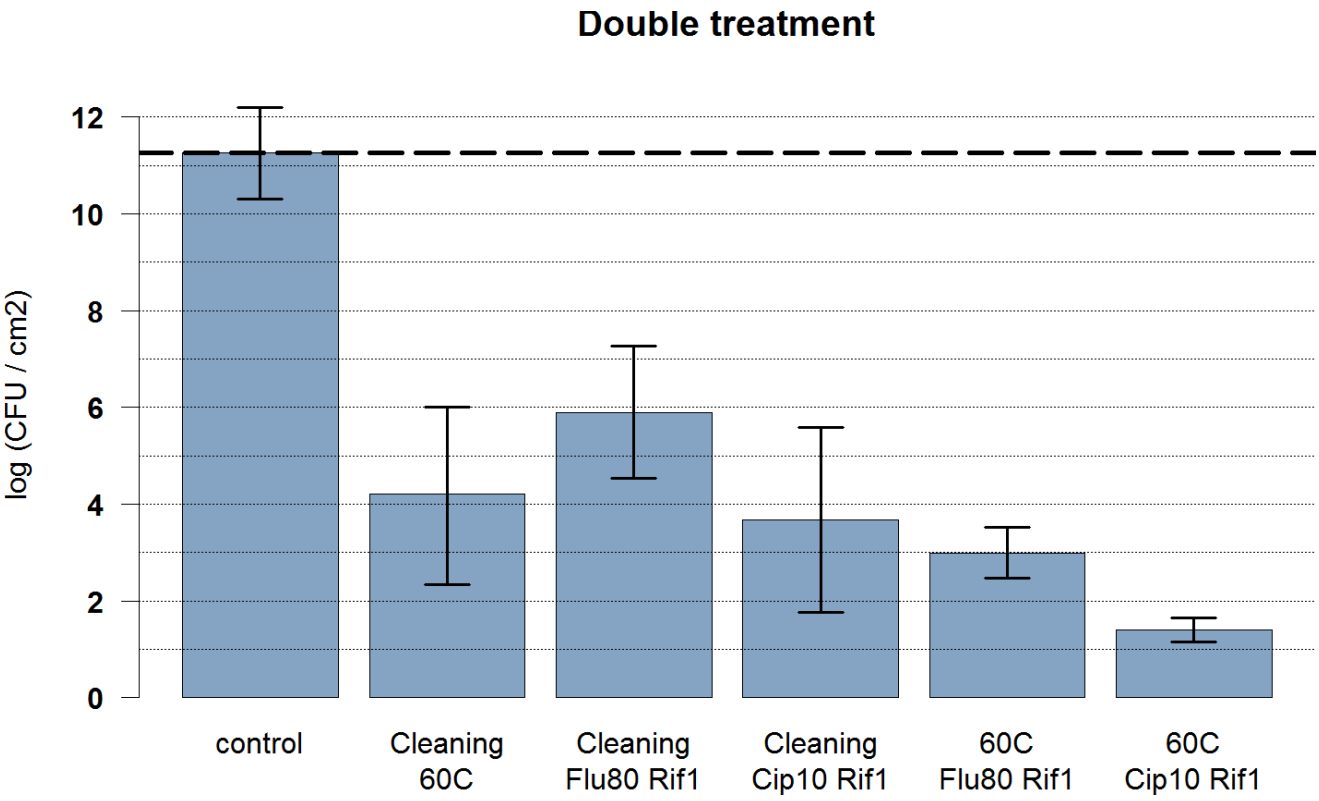

Fig. 4

Seven- to eight-day biofims: graph showing the log colony-forming units (CFUs) that remain for seven-day *Staphylococcus aureus* biofilms after a combination of two (double) treatments. The dashed line represents the control. Mean and 95% confidence intervals are presented. 'Cleaning' refers to mechanical cleaning. 60C, induction heating to 60°C for one minute; Cip10Rif1, ciprofloxacin 10 mg/l + rifampicin 1 mg/l; Flu80Rif1, flucloxacillin 80 mg/l + rifampicin 1 mg/l.

coupons. Regarding antibiotics, ciprofloxacin with rifampicin, and flucloxacillin with rifampicin, were the most effective at killing bacteria in the biofilm, with a 6.0 log and 6.1 log reduction in CFU/cm^2 respectively (Figures 2 and 3). Treatment with vancomycin, cefuroxime, and amoxicillin, all with rifampicin, gave 1.4 log, 4.8 log, and 3.6 log reduction in CFU/cm^2, respectively (Figure 2).

Mechanical cleaning resulted in 4.9 log reduction in CFU/cm^2, so a mean 2.3 × 10^6 CFU/cm^2 95% CI (1,584 to 3.7 × 10^9) remained on the coupons (Figure 3). Induction heating (60°C for one minute) resulted in 7.3 log reduction in CFU/cm^2, so a mean 9,770 CFU/cm^2 95% CI (7,943 to 11,749) remained on the coupons (n = 4, due to one contamination) (Figure 3).

Double combined treatments. The results of the double combined treatments are presented in Figure 4.

Induction heating (60°C for one minute) followed by 24 hours ciprofloxacin with rifampicin was the most effective double treatment, resulting in 9.9 log reduction in CFU/cm^2, so a mean of 25 CFU/cm^2 95% CI (14 to 45) remained on the coupons. This was a synergistic combination.

Induction heating (60°C for one minute), followed by 24 hours flucloxacillin with rifampicin, resulted in 8.3 log reduction in CFU/cm^2, so a mean of 977 CFU/cm^2 95% CI (295 to 3,311) remained on the coupons. This was an additive combination; it was not synergistic.

Mechanical cleaning followed by induction heating (60°C for one minute) resulted in a 7.1 log reduction in CFU/cm^2, so a mean of 1.6 × 10^4 CFU/cm^2 95% CI (219 to 1.0 × 10^6) remained on the coupons (n = 8, to account for possible contamination during the cleaning procedure, which did not occur). This was similar to induction heating alone (7.3 log reduction), so mechanical cleaning had no additional effect.

Mechanical cleaning followed by 24 hours ciprofloxacin with rifampicin resulted in a 7.6 log reduction in CFU/cm^2, so a mean of 4,786 CFU/cm^2 95% CI (59 to 3.9 × 10^5) remained on the coupons. This was an additive combination; it was not synergistic.

Mechanical cleaning followed by 24 hours flucloxacillin with rifampicin resulted in a 5.4 log reduction in CFU/cm^2, so a mean of 7.9 × 10^5 CFU/cm^2 95% CI (3.4 × 10^4 to 1.9 × 10^7) remained on the coupons. This was similar to flucloxacillin with rifampicin alone (6.1 log reduction), so mechanical cleaning had no additional effect.

Triple combined treatments. The results of the triple treatments are presented in Figure 5.

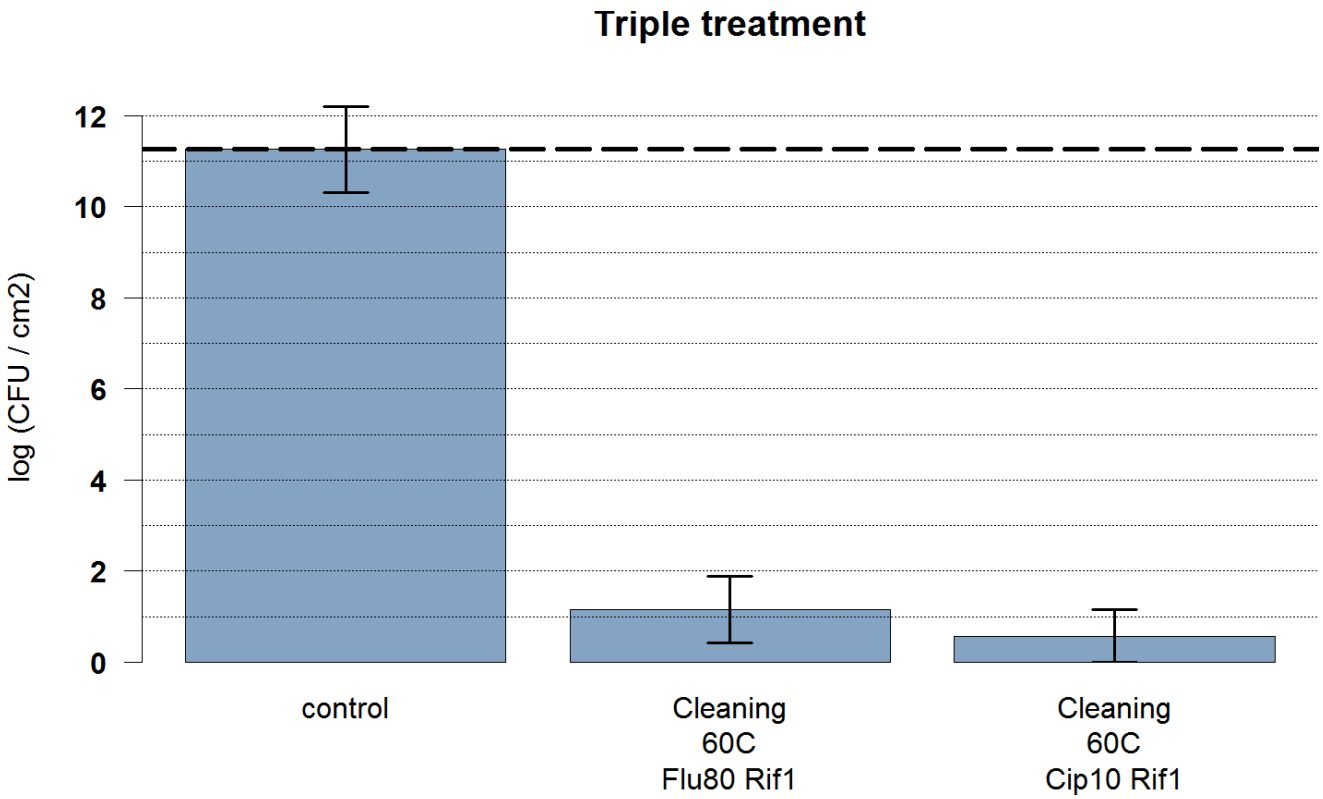

Fig. 5

Seven- to eight-day biofims: graph showing the log colony-forming units (CFUs) that remain for seven-day *Staphylococcus aureus* biofilms after combination of all three (triple) treatments. The dashed line represents the control. Mean and 95% confidence intervals are presented. 'Cleaning' refers to mechanical cleaning. 60C, induction heating to 60°C for one minute; Cip10Rif1, ciprofloxacin 10 mg/l + rifampicin 1 mg/l; Flu80Rif1, flucloxacillin 80 mg/l + rifampicin 1 mg/l.

Mechanical cleaning followed by induction heating (60°C for one minute), and subsequently followed by 24 hours of ciprofloxacin with rifampicin, resulted in 10.7 log reduction in CFU/cm^2, so a mean of four CFU/cm^2 95% CI (1 to 14) remained on the coupons.

Mechanical cleaning followed by induction heating (60°C for one minute), and subsequently followed by 24 hours of flucloxacillin with rifampicin, resulted in a 10.1 log reduction in CFU/cm^2, so a mean of 40 CFU/cm^2 95% CI (3 to 78) remained on the coupons.

Effect of each treatment. The random effect analyses showed that ciprofloxacin with rifampicin resulted in a mean 2.6 log reduction (95% CI 2.2 to 3.2) when used in combinations of treatments, compared to combinations of treatments not using ciprofloxacin and rifampicin. For flucloxacillin with rifampicin, there was a mean 1.5 log reduction (95% CI 0.1 to 2.9) when used in combinations of treatments, compared to combinations of treatments without these two antibiotics. For induction heating, there was a mean 2.9 log reduction (95% CI 1.6 to 4.2) when used in combinations of treatments, compared to combinations of treatments where this was not used. For mechanical cleaning, there was no apparent difference when used or not used in combinations of treatments (mean log reduction 0.7 (95% CI -0.3 to 1.7)).

14-day biofilm model. The results of the 14-day biofilm model are presented in Figure 6. Induction heating (60°C for one minute) followed by seven days of ciprofloxacin with rifampicin was the most effective combination: no viable bacteria could be detected after a week (of culturing) in three out of five experiments. The remaining two experiments had one CFU/cm^2 remaining. All treatments (either antibiotics alone or antibiotics after induction heating) showed the largest reduction in CFU/cm^2 in the first 24 hours, with a steady decline or stabilization during the remaining treatment period (seven days).

Discussion

Our results show that mechanical cleaning of the implant reduces the bacterial load by 4.9 log CFU/cm^2 in a laboratory-controlled, optimized scenario. This means that the bacterial load of the coupon (implant) is reduced by 99.999%, which can be considered a best-case scenario: in clinical reality, this level of reduction in bacterial load will probably not be achievable. Although the reduction in bacterial load was high in our study, there were 2.3 million bacteria per square centimetre remaining after mechanical cleaning. The biofilm will thus likely regrow from these remaining bacteria if no antibiotics are introduced. This temporary

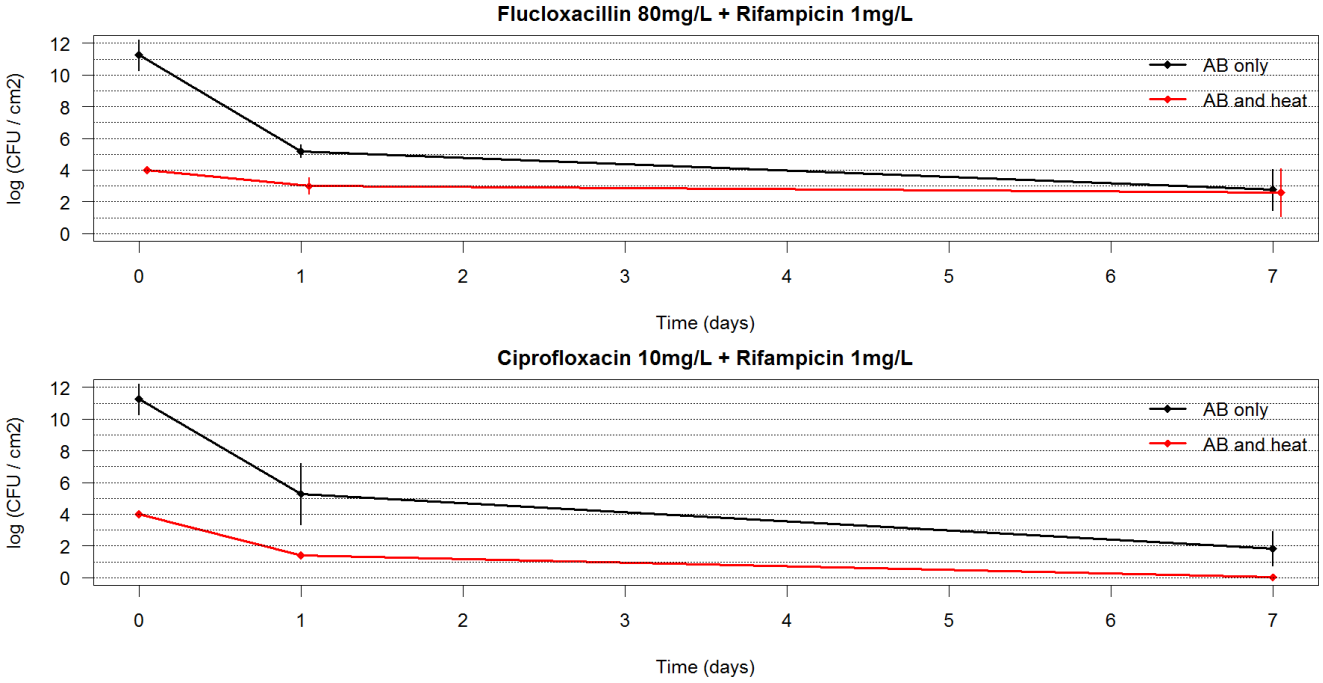

Fig. 6

Seven- to 14-day biofilms: graph showing the log colony-forming units (CFUs) that remain for seven-day *Staphylococcus aureus* biofilms after 24 hours, and seven-day exposure to antibiotics with (red) or without (black) induction heating to 60°C for one minute. The combination of induction heating and ciprofloxacin with rifampicin was synergistic, and this combination was the only one that fully eradicated the bacteria from the metal coupons. Means and 95% confidence intervals are presented. AB, antibiotics.

effect of mechanical cleaning of the metal surface was confirmed by the random effects model, which showed that the effect of mechanical cleaning was very limited when used in combination with other treatments: mean log reduction 0.7 (95% CI -0.3 to 1.7). While debridement is probably of importance for decreasing bacterial load from soft-tissue and fluids, antibiotics and NCIH are needed to eradicate the bacterial load from the metal surface of implants.

We should consider some limitations. First, although our results are based on a mature biofilm model of seven to 14 days old, these are still in vitro models and may not fully translate to in vivo scenarios. Physiological and molecular effects of thermal shock are not accounted for. Localized thermal shock or hyperthermia has been shown to increase blood flow, to increase blood vessel permeability, to activate the immune system, and to increase the permeability of membranes, all of which are expected to be helpful in curing PJI.[25] Second, the experimental model does not include peri-implant tissue, fluids such as plasma, haematoma, or human cells, and ignores the pharmacokinetics of daily administered antibiotics (and their metabolites). Although these limitations may be addressed in future in vitro experiments, they will probably require in vivo studies. Third, all experiments were performed with only a single strain of *S. aureus* (ATCC 29213). However, this strain is a biofilm-forming clinical isolate, and is validated for in vitro biofilm research related to PJI and DAIR.[14] Fourth, it is reported that *S. aureus* biofilms could reach a plateau in cell count after 14 days, which would make eradication of such fully developed biofilms even more challenging compared to seven-day biofilms.[26]

While mechanical cleaning removes bacteria, it does not weaken them or change their susceptibility to antibiotics. Induction heating, by contrast, killed 7.3 log of the bacteria in the biofilm and subsequently weakened the remaining bacteria, making them more susceptible to treatment with antibiotics: the combination of NCIH and ciprofloxacin with rifampicin was synergistic, and after this combination treatment no viable bacteria could be detected. The combination of induction heating and amoxicillin with rifampicin was also synergistic, and induction heating greatly improved the susceptibility of mature *S. aureus* biofilms to amoxicillin with rifampicin. A synergistic effect of antibiotics and induction heating has been previously described by Pijls et al[17] for *Staphylococcus epidermidis* biofilms. Hajdu et al[27] have shown that antibacterial activity of antibiotics was increased with heightened temperature. Similarly, Ricker and Nuxoll[21] have reported a synergistic effect of heat and erythromycin, tobramycin, and ciprofloxacin on *Pseudomonas* biofilms. The temperatures used in our study cause denaturation of several bacterial proteins, interfering with their function.[28] Wang et al[9] have suggested that NCIH may work by disruption of the bacterial membrane due to thermal damage which, in turn, could lead to bacterial death or bacteria being more susceptible to antibiotics. Therefore, the

combination of induction heating and antimicrobial therapy holds great promise.[6]

It is well known from clinical experience, as well as in vitro and in vivo research, that antibiotics alone are not sufficient for eradicating bacterial biofilms from implant surfaces.[1,14] Our results are in accordance with this observation, as mature biofilm survived seven-day exposure to antibiotics. Killing bacteria in the mature biofilm followed a specific pattern during seven-day antibiotic exposure: most bacterial death occurred during the first 24 hours, with a steady decline or stabilization during the remaining treatment period (seven days). We are not aware of any other in vitro studies that have exposed mature bacterial biofilms on titanium alloy coupons to seven days or more of antibiotics at concentrations that can be achieved clinically. It is thus important for future in vitro studies to consider exposing mature biofilms to longer (seven days or more) antimicrobial therapy, as this is more in line with weeks of clinical antibiotic treatment for patients with PJI.[1]

Importantly, killing bacteria with induction heating should not result in damaging the bone-implant or cement-implant interface by thermal necrosis, resulting in loosening of the implant. In our study, the maximal thermal dose was 60°C for one minute. Such temperature and duration is not uncommon during orthopaedic surgery, as it can be reached by using diathermia, drilling, inserting Kirschner wires, and cementing an implant.[29-32] Animal studies by Müller et al[33] have confirmed a lack of clinically relevant necrosis after induction heating of nickel-titanium-shaped memory rod in the femur of rats at 40°C to 60°C. The same research group has also heated osteosynthesis plates in a rabbit fracture model, and observed that all fractures healed.[34] Fang et al[35] have used induction heating to heat metal implants in a rat model up to 75°C without any significant thermal damage. Regarding soft-tissue, Chopra et al[13] have shown that thermal damage was confined to a region of less than 2 mm around the implant after heating it up to 80°C to 100°C with induction heating. It is also possible during DAIR to use special heating techniques, such as segmental induction heating, to selectively heat only a segment of an implant while using the remainder of the implant as a heat sink.[11] This technique could help avoid thermal damage to areas vital for implant fixation.

In conclusion, mechanical cleaning, antibiotics, and non-contact induction heating all reduced the bacterial load of mature *S. aureus* biofilms with approximately 5 log or more as a single treatment. The effect of mechanical cleaning on mature *S. aureus* biofilms was limited when used in combination with antibiotics and/or induction heating. The combination of NCIH and ciprofloxacin with rifampicin was synergistic and after this combination treatment no viable bacteria could be detected.

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