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Research Article

RESEARCH ARTICLE

# Preliminary results of a new antibiotic susceptibility test against biofilm installation in device-associated infections: the Antibiofilmogram®

Jason Tasse[1,2,*], Delphine Croisier[3], Stéphanie Badel-Berchoux[1], Pascal Chavanet[3,4], Thierry Bernardi[1], Christian Provot[1] and Frédéric Laurent[2]

[1]BioFilm Control, Saint-Beauzire 63360, France, [2]French National Reference Centre for Staphylococci, International Centre for Infectiology Research – Inserm U1111, Hospices Civils de Lyon, Lyon 69000, France, [3]Vivexia Laboratory, Dijon, France and [4]Department of Infectious Disease, Dijon University Hospital, Dijon 21000, France

*Corresponding author: Laboratoire de Bactériologie, Groupement Hospitalier Nord; 103 Grande Rue de la Croix-Rousse, Lyon 69004, France. Tel: +33-472-003-770; E-mail: tasse.jason@gmail.com

**One sentence summary:** Preliminary results of a new test called Antibiofilmogram® able to identify the capacity of antibiotics to inhibit biofilm formation.

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## ABSTRACT

Biofilms are complex communities of microorganisms embedded in an extracellular matrix and adherent to a surface. The development was described as a four-stage process leading to the formation of a mature biofilm which was resistant to immune system and antibiotic actions. In bone and joint infections (BJIs), the formation of biofilms is a leading cause of treatment failure. Here we study the capacity of 11 antibiotics commonly used in the treatment of BJIs to inhibit the biofilm formation on 29 clinical *Staphylococcus aureus* isolates by a new test called Antibiofilmogram®. The minimal inhibitory concentration (MIC) and biofilm MIC (bMIC) were determined *in vitro* and showed similar values for clindamycin, fusidic acid, linezolid and rifampin. Reversely, daptomycin, fosfomycin, gentamicin and ofloxacin showed a bMIC distribution different from MIC with bMIC above breakpoint. Finally, cloxacillin, teicoplanin and vancomycin revealed an intermediate bMIC distribution with a strain-dependent pattern. A murine *in vivo* model of catheter-associated *S. aureus* infection was made and showed a significant reduction, but not total prevention, of catheter colonization with cloxacillin at bMIC, and no or limited reduction with cloxacillin at MIC. Antibiofilmogram® could be of great interest after surgical operations on contaminated prostheses and after bacteremia in order to prevent the colonization of the device.

**Keywords:** inhibition; biofilm; bone; prevention; BJIs; antibiotic; bMIC

## INTRODUCTION

Biofilm is classically defined as a group of microorganisms attached to a surface and embedded in a self-produced polymeric matrix composed of extracellular DNA, proteins and polysaccharides (Izano *et al.* 2008; McCarthy *et al.* 2015). This mode of growth provides protection to bacteria from the immune system and drug treatment due to (i) poor antibiotic penetration, (ii) the formation of persister cells in the deeper biofilm layers and (iii) the slow rate of bacterial growth due to the

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physiological state (dormant, inactive, stress adaptation) (Stewart 2015) and the microenvironment (gradients of nutrients and oxygen). Bacterial biofilm is widely found in most human diseases and especially in patients with indwelling devices including intravenous catheter, vascular prosthesis or orthopedic devices (Song *et al*. 2013; Wu *et al*. 2015).

In bone and joint infections (BJI), *Staphylococcus aureus* is the main prevalent organism isolated and accounts for more than 50% of infections (Peel *et al*. 2012; Kremers *et al*. 2015). These bacteria are recognized as being high biofilm producers, leading to considering BJIs as very difficult to treat diseases, associated with high morbidity and high cost (Kurtz *et al*. 2012; Jacqueline and Caillon 2014; Gbejuade, Lovering and Webb 2015). The management of BJIs in the presence of an infected prosthetic device requires both a surgical procedure and long-term antimicrobial chemotherapy (Osmon *et al*. 2013). Acute infections (diagnosed within 4 weeks after the initiation of symptoms) and early infections (occurring within the first 4 post-operative weeks) are classically treated with implant retention (Jiranek *et al*. 2015). Inversely, the recommended surgical treatment for chronic and tardive infections is a one or two-stage exchange of the device, with removal of necrotic tissue, bone cement and prosthetic material to reduce most of the bacterial burden. Despite optimal medical care, chronic evolution and relapse in infection are frequently reported. Indeed, the residual bacteria can recolonize the prosthetic device, especially in one-stage exchange surgery, and an early biofilm can be reconstituted. The inhibition of bacterial adhesion to the material, which corresponds to the first step of the biofilm formation, may be a good target to avoid treatment failure.

The standard method used in laboratories to determine the antibiotic susceptibility of microorganisms is the minimal inhibitory concentration (MIC) which is only based on the planktonic behavior of bacteria. The minimum biofilm eliminating concentration is a reliable method for biofilm antimicrobial susceptibility testing but the values only reflect the antibiotic efficiency on a mature biofilm which are often more than 1,000-fold higher than the MICs and therefore not adaptable for clinical use (Molina-Manso *et al*. 2013). Moreover, this method is long and fastidious and not suitable for routine clinical use. To date, there is no method designed to evaluate the biofilm prevention capacity of antibiotics.

The BioFilm Ring Test® (BioFilm Control, Saint-Beauzire, France) is a method allowing for the measurement of the initial steps of biofilm formation, which is the ability of bacteria to adhere to an inert surface and to initiate matrix production (Chavant *et al*. 2007). This method is based on the immobilization of magnetic beads present in the culture media and can be used to study the capacity of antibiotics to prevent biofilm installation. Here we evaluated the capacity of antibiotics commonly used in the treatment of BJIs to inhibit biofilm formation on 29 clinical *S. aureus* isolates by a new test called the Antibiofilmogram®.

The proof of concept of this method was then explored for cloxacillin in a murine *in vivo* model of catheter-associated *S. aureus* infection. In this study, we investigated whether exposure above the biofilm MIC (bMIC) could prevent biofilm formation according to the Antibiofilmogram® results.

## MATERIALS AND METHODS

### Materials

Antibiotics were purchased from EDQM (Strasbourg, France) except daptomycin and linezolid from Sigma Aldrich (Saint-Quentin-Fallavier, France). E-test®, Colombia blood agar and Chapman plates were purchased from bioMérieux (Marcy l'Etoile, France). Brain heart infusion (BHI) broth was from Conda (Madrid, Spain). 96-well microplates were from Corning (Amsterdam, The Netherlands). The microbeads and contrast liquid were manufactured by BioFilm Control. The plate reader (scanner), the block carrying 96 micromagnets and the BFC Elements® software (first version) were developed by BioFilm Control. Sterile polyurethane catheters were from Teleflex Medical (Le Faget, France).

### Collection of *Staphylococcus aureus* from BJI

A collection of 29 *Staphylococcus aureus* isolates (LYO-SXX) responsible for a first episode of BJI in the Hospices Civils de Lyon hospitals (Lyon, France) from 2001 to 2010 was used in this study. The French South-East ethics committee approved this collection of clinical data and clinical strains (reference number 2013–018). All strains were characterized for biofilm formation by the crystal violet assay. According to the classification of Stepanovic *et al*. (2000), 1/29 strain was categorized as weakly adherent, 15/29 were categorized as moderately adherent and 13/29 were categorized as strongly adherent.

### MIC determination

MICs of cloxacillin, daptomycin, fosfomycin, gentamicin, linezolid, ofloxacin, rifampin, teicoplanin, vancomycin, fusidic acid and clindamycin were determined using the E-test® method. All isolates were subcultured on Colombia blood agar plates and incubated at 37°C for 18 h and the inoculum was standardized to 0.5 McFarland. The MIC values were determined after 18 h of incubation at 37°C according to the manufacturer's instructions. The S. *aureus* ATCC 25923 was used as a control for MICs determination. The results were interpreted according to the Comité de l'Antibiogramme de la Société Française de Microbiologie 2015 (CASFM) guidelines.

### bMIC determination

The bMIC of clindamycin, cloxacillin, daptomycin, fosfomycin, fusidic acid, gentamicin, linezolid, ofloxacin, rifampin, teicoplanin and vancomycin were determined using the new Antibiofilmogram® test (BioFilm Control). The isolates were subcultured on Colombia blood agar plates and then grown in BHI at 37°C for 18 h. After standardization of the inoculum to $OD_{600\ nm} = 1 \pm 0.05$ (Ultrospec 10 Cell Density Meter, Amersham Biosciences, USA) the bacteria were diluted in sterile BHI to obtain a final concentration of $4 \times 10^6$ UFC $mL^{-1}$. This BHI which also contained 10 $\mu$L $mL^{-1}$ of 'Toner', consisting of a magnetic bead suspension, was added at 200 $\mu$L per well to a 96-well microplate containing antibiotic solutions. Dedicated BHI provided in the kit by the manufacturer was used because it ensured the growth and the measurement of biofilm formation of most bacteria and has no interaction with magnetic beads. The microplates were pre-loaded by 20 $\mu$L of antibiotic solutions in a range of eight 2-fold dilutions ranging from 8 to 0.0625 $\mu$g $mL^{-1}$ for daptomycin, fusidic acid, gentamicin and rifampin; 16 to 0.125 $\mu$g $mL^{-1}$ for clindamycin, cloxacillin, linezolid, teicoplanin and vancomycin; 64 to 0.5 $\mu$g $mL^{-1}$ for ofloxacin and 128 to 1 $\mu$g $mL^{-1}$ for fosfomycin. The microplate containing bacteria and antibiotics was incubated at 37°C for 4 h. The wells were covered with 100 $\mu$L of a contrast liquid solution before being scanned a first time by the plate reader. The microplate was then placed for 1 min on a magnet support and scanned a second time. During magnet contact, free beads were attracted to the center of each well,

resulting in a visible spot. In contrast, beads trapped in a biofilm were unable to move and no spot was formed. For each plate, the wells in column 1 were used for both negative control (BHI plus beads to check for the absence of contamination, leading to a spot) and positive control (BHI plus beads plus strain to check for the strain's capacity to form a biofilm, leading to an absence of spots). The spot features were read visually for each antibiotic to quantify the efficiencies to prevent biofilm formation. A visible spot meant that the concentration of antibiotic tested was able to prevent biofilm formation. The bMIC was determined for each antibiotic as the lowest concentration where a spot, similar to negative control, was visible. As the aim of this method was to measure the initial step required for biofilm formation, and as it has been well demonstrated that if this step was inhibited, biofilm formation can no longer occur, we used 4 h incubation time to test the antibiotic action in our study. To be sure that the toner has no interaction with antibiotics, two microplates with or without magnetic beads were performed for the *S. aureus* ATCC 29213 strains as described above. Microplates were incubated for 24 h at 37°C, and the MIC was determined for each antibiotic.

## *In vivo* model

### *Animals*

BALB/c female mice (weighing 22–24 g) were used for all studies (Charles River, France). These animals were immunocompetent and housed in a protected area in the 'Centre de Zootechnie de l'Université de Bourgogne' (Biosafety level 2 facility) and were fed ad libitum according to the current recommendations by the European Institute of Health. No fasting period was performed in this study. Before each experiment, the animals were stabulated for a week in a conventional area of the animal facility. During this period and for the duration of the study, qualified members of staff checked on the animals twice a day and assessed their well-being. The animal facility is authorized by the French authorities (Agreement N°C 21 464 04 EA). Animal housing and experimental procedures were performed according to the French and European Regulations and the NRC Guide for the Care and Use of Laboratory Animals. All the procedures using animals were submitted to the Animal Care and Use Committee C2EA approved by the French authorities.

### *Experimental model of infection*

Of the 29 *S. aureus* strains, three (LYO-S31, LYO-S39 and LYO-S14) were used for this *in vivo* study because the differences between MIC and bMIC values of cloxacillin were significant.

The animals were anaesthetized by intraperitoneal (ip) injection of a mixture of ketamine (50 mg $kg^{-1}$) and xylazine (10 mg $kg^{-1}$). The flanks were shaved and then disinfected (three cycles of betadine). A subcutaneous incision of 0.2 cm was performed under sterile conditions and a 1 cm segment of polyurethane catheter (Ref ES-04730 Arrow international) cut into two longitudinal fragments was inserted subcutaneously (at about 2 cm from the incision to avoid any contamination). Inoculation was performed simultaneously by placing 50 $\mu$l of the bacterial culture onto the catheter ($10^7$ CFUs per mouse). The incision was sutured and disinfected. In each study, non-infected mice were used to guarantee that the experiment was performed in sterile conditions.

### *Drug pharmacokinetics*

Cloxacillin was selected as a good candidate for the *in vivo* investigation as T > MIC is the most relevant pharmacodynamic parameter for beta-lactam. Cloxacillin could be administered in order to mimic a continuous infusion exposure. In order to determine the spontaneous pharmacokinetic (PK) of cloxacillin, a previous single dose study was performed with mice given cloxacillin intraperitoneally at doses of 100, 200 and 400 mg $kg^{-1}$. Pure commercial solution was used for Cloxacillin PanPharma 1 g and reconstituted in sterile serum saline according to the manufacturer's instructions.

The mice were anaesthetized by gas (air/isoflurane mix) in order to collect blood by cardiac puncture. All the animals were then euthanized by cervical dislocation. The blood samples were centrifuged (10 min at 2300 g, 4°C) within 60 min of collection and serum samples were stored at −80°C with all samples frozen within 1 h of processing. Drug levels in serum were determined using an HPLC-DAD method, according to the ISO 15189 norm (Dr AS Lemaire-Hurtel, Laboratoire de Pharmacologie et Toxicologie, CHU Amiens, France). The lower limit of quantification was 2.5 $\mu$g $mL^{-1}$.

After PK characterization of the single doses, these data were used to determine the optimal regimen that should be used to simulate a cloxacillin concentration time profile between MIC and bMIC or greater than bMIC: (i) between 0.125 and 2 mg $L^{-1}$ or greater than 2 mg $L^{-1}$ for LYO-S14 infected mice; (ii) between 0.39 and 4 mg $L^{-1}$ or greater than 4 mg $L^{-1}$ for LYO-S31 infected mice; (iii) between 0.19 and 4 mg $L^{-1}$ or greater than 4 mg $L^{-1}$ for LYO-S39 infected mice.

The continuous equivalent treatment of cloxacillin was initiated intraperitoneally in infected mice 30 min before the infection in order to study the capacity of biofilm inhibition (this timing corresponded to the $T_{max}$ obtained in mice in a previous pilot study) and included (i) a loading dose ($T_{-0h30}$) at 30 mg $kg^{-1}$ (>bMIC) or 10 mg $kg^{-1}$ (<bMIC) and four lower doses administered every 2 h ($T_{1h30}$, $T_{3h30}$, $T_{5h30}$ and $T_{7h30}$ post-infection) at 15 mg $kg^{-1}$ (>bMIC) or 3 mg $kg^{-1}$ (<bMIC). Overall, five doses were administered to cover a 10 h exposure period (meaning 30% of the time over 24 h). The mice were then anesthetized and euthanized by cervical dislocation at 12, 24 or 30 h post-treatment. The catheter was removed, transferred to a sterile tube and processed for bacterial quantification.

Confirmatory studies were also undertaken in treated but non-infected animals (10 mice in the >bMIC treated group and 10 mice in the <bMIC group) to ensure that target exposures of cloxacillin were achieved.

## Bacterial quantification

Each fragment of catheter was individually washed under aseptic conditions in an Eppendorf tube (three successive washing steps with 300 $\mu$L of sterile saline) to remove non-adherent bacteria. After the last wash, the catheter was resuspended in 1 mL of sterile saline and was vigorously vortexed for 30 s before putting in an ultrasonic bath (Advantage Lab) for 3 min, 50 Hz at room temperature in order to detach all adherent bacteria from the catheter. The bacterial burden was measured by plating several successive dilutions of this suspension onto Chapman agar plates for 48 h at 37°C.

## Statistical analysis

*In vivo* data were analyzed by a Mann-Whitney analysis using Prism software (GraphPad, San Diego, USA). Median and standard deviations are shown and *P* values are NS: not significant, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.0001$.

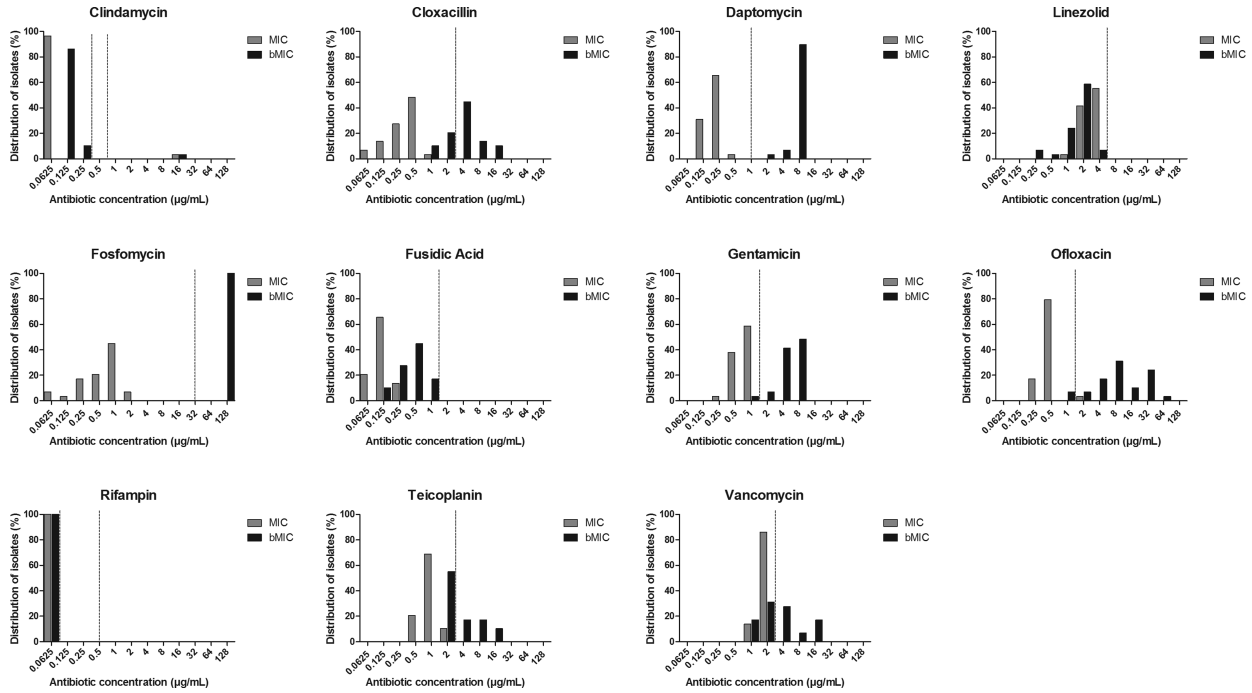


**Figure 1.** MIC (gray bars) and bMIC (black bars) distribution of 29 clinical isolates involved in BJIs for 11 antibiotics. Results are expressed as a percentage. MICs were determined using the E-test® according to the manufacturer's instructions. The breakpoint defined by the CASFM is presented for each antibiotic using dotted lines. bMICs were determined using Antibiofilmogram®. For this method, the inoculum of each isolate was standardized and diluted in BHI containing toner (magnetic beads). The suspension was added to a 96-well microplate with antibiotics tested in a range of eight 2-fold dilutions. After incubation at 37°C for 4 h, the microplate was placed on a magnet support to evaluate the mobility of the beads in order to deduce the capacity of each concentration of antibiotic to inhibit biofilm formation.

# RESULTS

## MIC and bMIC determination

Susceptibility to 11 antibiotics for 29 clinical isolates involved in BJI was determined using the E-test method (Fig. 1). Only two isolates showed resistance to ofloxacin and clindamycin, respectively. For all the other antibiotics, the strains were classified as susceptible.

The capacity of the antibiotics to prevent biofilm formation was evaluated using the Antibiofilmogram® method. Antibiotic was present from the initial incubation time (H0). After 4 h of incubation using BHI provided by the manufacturer without antibiotics (positive control), all the strains were able to block beads which means they formed an early biofilm. No interaction was observed after 24 h incubation between magnetic beads and antibiotics using the ATCC 29213 strains. In the presence of antibiotics, three profiles of biofilm prevention based on bMIC were identified. Clindamycin, fusidic acid, linezolid and rifampin revealed a bMIC distribution similar or close to the MIC and at least, a bMIC value below breakpoint(s) for all isolates, except the strain resistant to clindamycin. On the contrary, daptomycin, fosfomycin, gentamicin and ofloxacin showed a bMIC distribution different from the MIC values with bMIC above breakpoint for all isolates. Finally, cloxacillin, teicoplanin and vancomycin revealed an intermediate bMIC distribution with part of the population harboring a bMIC over breakpoint. Indeed, 9, 16 and 14 strains showed a bMIC below breakpoint for these three drugs, respectively.

## *In-vivo* model of catheter-associated infection

To support *in vitro* results and evaluate the predictive value of Antibiofilmogram® for the *in vivo* biofilm prevention capacity of antibiotics, three *Staphylococcus aureus* strains were chosen to be tested in a mouse model of catheter-associated infection, treated with cloxacillin prior to and following the inoculation of the strain. Cloxacillin was the first antibiotic tested in this model because the bMIC/MIC ratio was high (16, 10.3, 21.1 for these three strains, respectively; (Table 1)). This difference between MIC and bMIC allowed us to simulate exposure within or above these concentrations. The average cloxacillin serum concentrations obtained after a >bMIC simulation was about 8 $\mu$g mL$^{-1}$ (except after the loading dose where the Cmax was about 14 $\mu$g mL$^{-1}$) and the average cloxacillin serum concentrations obtained after a <bMIC simulation was 2 $\mu$g mL$^{-1}$ (except after the loading dose where the Cmax was about 7 $\mu$g mL$^{-1}$).

The results for the isolate LYO-S14 revealed that there is no significant difference in colonization based on adherent bacteria enumeration between control and MIC concentrations after

**Table 1.** MIC, bMIC and bMIC/MIC ratio of cloxacillin obtained for the isolates LYO-S14, LYO-S31 and LYO-S39. Results are expressed as a concentration ($\mu$g mL$^{-1}$). MICs were determined using the E-test, and bMICs were determined using the Antibiofilmogram.

| | Cloxacillin | | |
|---|---|---|---|
| Strain number | MIC ($\mu$g mL$^{-1}$) | bMIC ($\mu$g mL$^{-1}$) | bMIC/MIC ratio |
| **14** | 0.125 | 2 | 16 |
| **31** | 0.39 | 4 | 10.3 |
| **39** | 0.19 | 4 | 21.1 |

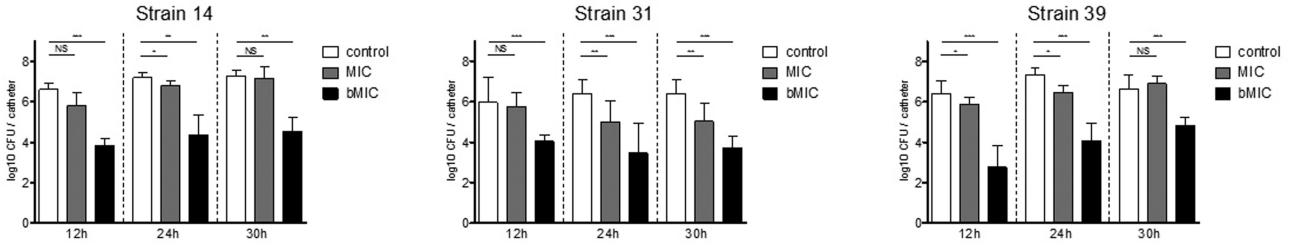


**Figure 2.** Cloxacillin efficiency in biofilm prevention in *in vivo* model. The catheter infection model was performed on strains LYO-S14, LYO-S31 and LYO-S39. Cloxacillin was administered at two concentrations targeting the MIC (gray bars) and bMIC (black bars) as described in materials and methods section. White bars represent the control mice without antibiotic treatment. Quantification of adherent bacteria on the implanted polyurethane catheter was measured after 12, 24 or 30 h of treatment by serial dilution and plate counting on agar plates. Results are expressed in log10 CFU/catheter. Statistical differences $^{*}(P < 0.05)$, $^{**}(P < 0.01)$ and $^{***}(P < 0.001)$ between each group were obtained using Mann-Whitney U test.

12 or 30 h of treatment (Fig. 2). On the contrary, the bMIC concentration of cloxacillin significantly reduced the colonization of the catheter, showing a 2.7 log ($P < 0.001$), 2.8 log ($P < 0.01$) and 3.1 log ($P < 0.01$) reduction of adherent bacteria quantification after 12, 24 and 30 h, respectively (Fig. 2). Similar results were observed with the strain LYO-S39, showing a high reduction in colonization with bMIC concentrations (3.5 log, 3.0 log and 1.9 log after 12, 24 and 30 h, respectively; $P < 0.001$) and a lower or non-significant reduction with MIC concentrations (0.5 log ($P < 0.05$), 0.6 log ($P < 0.05$) and no reduction after 12, 24 and 30 h, respectively). Finally, the isolate LYO-31 showed a higher diminution of colonization with bMIC than with MIC, showing a reduction of 2.5 log ($P < 0.001$) vs 0.6 log (NS), 2.9 log ($P < 0.001$) vs 1.4 log ($P < 0.01$) and 2.7 log ($P < 0.001$) vs 1.3 log ($P < 0.01$) after 12, 24 and 30 h respectively.

## DISCUSSION

It has been estimated that 65% of nosocomial infections are biofilm associated. Bacteria embedded in biofilms are protected against the action of most of antibiotics. For example, BJIs involving biofilms, especially in the presence of a foreign body, are very difficult to treat and require intensive management including in most cases, the removal of the infected device. To fight against biofilms, strategy developments are mainly focused on how to kill bacteria on already formed biofilms. Here, we proposed a new vision of how antibiotics can be selected to have the best profile of antimicrobial activity including biofilms. Indeed, the goal of this study was not to determine antibiotic efficiency against mature biofilms but to evaluate the ability of antibiotics to prevent biofilm formation. This could be of great interest by reducing the incidence of BJIs in a context where prosthesis implantations continue to rise. Here, we evaluated the bMIC of 11 antibiotics on 29 clinical isolates.

In order to study the action of antibiotics on growth inhibition and biofilm prevention, both the classical MIC and the new bMIC values were determined using the E-test and the Antibiofilmogram® methods, respectively. Fosfomycin is the only antibiotic that showed no efficacy against biofilm prevention, with bMICs $> 128$ $\mu$g mL$^{-1}$ for all isolates. Moreover, 26/29 (90%) isolates have a bMIC for daptomycin of 8 $\mu$g mL$^{-1}$. Conversely, clindamycin and rifampin showed high potency of biofilm prevention with low bMIC for all isolates, especially for rifampin for which all isolates have a bMIC $< 0.0625$ $\mu$g mL$^{-1}$. These results support the hypothesis that these antibiotics have a similar potency of biofilm prevention against all *Staphylococcus aureus*. To validate this hypothesis, further studies including larger numbers of strains are needed. Alternatively, cloxacillin, linezolid, fusidic acid, gentamicin, ofloxacin, teicoplanin and vancomycin showed different bMICs for the 29 isolates. Indeed, the bMIC ranged from four or more antibiotic 2-fold dilutions. These results showed that antibiotic susceptibility in terms of biofilm prevention depends on the isolate.

The bMIC determined in this study was always higher than the MIC for all the antibiotics. Indeed, the bMIC ranged from 0.125 to 0.25 $\mu$g mL$^{-1}$; 0.125 to 1 $\mu$g mL$^{-1}$; 0.25 to 4 $\mu$g mL$^{-1}$ and 0.0625 $\mu$g mL$^{-1}$ for clindamycin, fusidic acid, linezolid and rifampin, respectively. PK studies have shown that these antibiotics have a good diffusion in bone tissue (Cluzel *et al.* 1984; Turnidge 1999; Lovering *et al.* 2002; El Samad *et al.* 2008) suggesting that bMIC could be reached in infected patients.

*In vivo* results have shown no or limited reduction in adherent bacteria when simulating a <bMIC, but >MIC exposure. Conversely, when simulating a >bMIC exposure, even if a complete prevention was not observed as expected according to *in vitro* results, a highly significant reduction in biofilm formation was obtained (at least 1.9 log). The differences in the *in vivo* and *in vitro* results are likely related to the higher size of the inoculum in mice ($10^7$ CFUs per mouse) compared to the inoculum used *in vitro* ($10^6$ CFUs per wells). To confirm a complete protection of the device, further studies deserved to be done using a lower inoculum, as previously reported by Metsemakers *et al.* (2015). The different nature of the devices, i.e. polystyrene for wells used *in vitro* and polyurethane for catheters used *in vivo* might have also impact the differential ability to form biofilm in presence of antibiotics. Finally, exposure to antibiotics was also different. For Antibiofilmogram®, concentrations are fixed through the *in vitro* experiment. Conversely, if the target of exposure for cloxacillin was obtained *in vivo*, these are total serum concentrations. Neither the free fraction nor the tissue penetration or its variations at the site of infection were taken into account. These points should be addressed. In the future, other animal models, more complex but likely more relevant, could be useful, including rabbit prosthetic-infected implant model with monitored antibiotic delivery (human-like continuous infusion) (Isiklar *et al.* 1996; Sheehan *et al.* 2004).

In this study, we only tested the efficiency of cloxacillin *in vivo*. It could be interesting to evaluate the capacity of other antibiotics that are inefficient in biofilm prevention according to Antibiofilmogram®, such as fosfomycin. Indeed, it could be anticipated that the fosfomycin regimen would be associated to poor efficacy in biofilm prevention, even at high concentrations, which may confirm that the efficiency of the bMIC of cloxacillin was not solely due to the higher concentration of drugs administered.

Finally, only three strains were tested *in vivo* and all the isolates included in this study were isolated from BJIs. It could be of great interest to include *S. aureus* strains from other biofilm infections as well as methicillin-resistant *S. aureus* to study the antibiotic efficiency on resistant strains. However, BJIs are mainly caused by staphylococci, and biofilms play a key role in the management of these infections, supporting the clinical relevance of this study (Arciola *et al.* 2015).

The current clinical guidelines for orthopedic infection devices are only based on the MIC. Despite the administration of antibiotics classified as susceptible on the basis of classical antimicrobial susceptibility tests and optimal management of infection, treatment failures are reported (Jover Saenz *et al.* 2007; Lora-Tamayo *et al.* 2013). The combination of MIC and bMIC could help the clinician to optimize antibiotic therapy and to select drugs showing the best antimicrobial profile, i.e. active on both planktonic and sessile bacteria. Within clinical strains classified as susceptible regarding MIC, the Antibiofilmogram® would help to discriminate in routine the strains able to form biofilm in the presence of antibiotics (classified as resistant regarding bMIC) from the strain not able to form biofilm in the presence of antibiotics (classified as susceptible regarding bMIC).

In the presence of material, it has been shown that only $10^2$ CFU $mL^{-1}$ of *S. aureus* are sufficient to induce an infection (Zimmerli *et al.* 1982). After surgical treatment, this amount of bacteria can be kept. The prosthetic material remaining in place in case of debridement as well as new material during one-stage protocol can be recolonized by residual bacterial inoculum leading to treatment failure (Marculescu *et al.* 2006; Cobo *et al.* 2011; Tornero *et al.* 2012). *In vivo* results revealed that a high log reduction of adherent bacteria can be obtained using the bMIC of cloxacillin. In this context, Antibiofilmogram® could be useful when the bacteria was identified from preoperative biopsy (blood culture, synovial fluid aspiration, true cut sampling performed prior surgery) and could permit to adjust pre-operative and early post-operative antibiotherapy. By preventing colonization of the device, the bMIC could facilitate the immune system to eliminate the remaining bacteria before the reoccurrence of biofilm and avoid relapse. Such new approach and data are of major interest because surgical procedure with only one surgery is less intensive, costs significantly less and may provide outcomes superior to two-stage exchanges (Lorenze *et al.* 1998; Nagra *et al.* 2015). In order to be more representative of the guidelines in the management of BJI, combination therapies should be tested. Indeed, antibiotics are never administered alone. The efficiency of combination therapies is known to be significantly better than antibiotic monotherapies (Zimmerli, Trampuz and Ochsner 2004). Further studies are needed to estimate the bMIC of antibiotic combination, especially with rifampin which is considered to be a key molecule for the treatment of biofilm-associated infection (Zimmerli *et al.* 1998). To finish, the bMIC could be useful for patients with prosthetic devices who develop *S. aureus* bacteremia. Indeed, the risk of the prosthesis becoming infected is high and can occur in nearly half of patients (Murdoch *et al.* 2001; Chu *et al.* 2005). Administration of antibiotics able to reduce the risk of colonization could be interesting in terms of cost, morbidity and mortality by reducing the number of patients infected.

To our knowledge, there are no studies that investigated the capacity of antibiotics to inhibit biofilm formation. Further studies were needed to confirm these preliminary results of Antibiofilmogram®. Finally, the determination of bMIC could be applied to a large variety of infections with medical devices such as drug delivery systems, prosthetic vascular grafts or urinary catheters as well as cystic fibrosis.

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***Conflict of interest.*** None declared.

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