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1 Differential response to antibiotic therapy in staphylococcal infective endocarditis: contribution of an
2 *ex vivo* model

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25 Abstract

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27 Staphylococcal infective endocarditis (IE) remains a hard-to-treat infection with high mortality. Both
28 the evaluation of new innovative therapies and research on alternative models mimicking human IE
29 are therefore urgently needed to improve the prognosis of patients with diagnosed IE. Dalbavancin is
30 a novel anti-staphylococcal lipoglycopeptide but there is limited data supporting its efficacy on biofilm
31 infections. This antibiotic could be an alternative to current therapies for the medical treatment of IE
32 but it needs to be further evaluated.

33 Here we developed an original *ex vivo* model of *Staphylococcus aureus* IE on human heart valves and
34 assessed biofilm formation on them. After validating the model, the efficacy of two antistaphylococcal
35 antibiotics, vancomycin and dalbavancin, was compared by measuring and visualizing their respective
36 ability to inhibit and eradicate late-formed biofilm. Determination of the minimum biofilm inhibitory
37 (MbIC) and eradicating (MbEC) concentrations in our *ex vivo* model identified dalbavancin as a
38 promising drug with much lower MbIC and MbEC than vancomycin (respectively <0.01 mg/L versus 28
39 mg/L and 0.03 mg/L versus 32 mg/L)

40 This data highlights a strong bactericidal effect of dalbavancin particularly on an infected heart valve
41 compared to vancomycin. Dalbavancin could be a realistic alternative treatment for the management
42 of staphylococcal IE.

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44 Introduction

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47 *Staphylococcus aureus* is the leading cause of Infective endocarditis (IE), and accounts for 40%
48 of cases of IE¹. *S. aureus* IE is characterized by the occurrence of early heart valve damage, and is
49 associated with a high mortality rate^{2,3}. The pathogenesis of IE leads to the formation of vegetations
50 on damaged heart valves, where adherent bacteria, platelets, immune cells and fibrin aggregate⁴. *S.*
51 *aureus* develops a biofilm inside vegetations with decreased metabolic activity⁵. Biofilm plays an
52 important role in the difficulties encountered when treating staphylococcal endocarditis via three main

53 effects: i) poor antibiotic penetration inside the biofilm, ii) decreased bacterial metabolic status leading
54 to reduced antibiotic activity; iii) biofilm detachment leading to secondary localizations and/or
55 thromboembolic events. As Staphylococci regularly colonize human skin, they are particularly involved
56 in health-care associated IE, with a prevalence of both hospital and community-acquired methicillin-
57 resistant isolates (MRSA) reaching 43 % in some European countries in 2021⁶ while community-
58 acquired MRSA has become predominant in North America⁷.

59 Despite improvements in diagnosis, management tools and surgery, in-hospital mortality of patients
60 with IE remains stable at 20%¹. Current international guidelines recommend vancomycin (VAN) as the
61 first-line therapy for MRSA native (monotherapy) and prosthetic valve (combination) IE^{8,9}. Long half-
62 life lipoglycopeptides such as dalbavancin (DAL) could be of interest, with evidence of its antibiofilm
63 activity in preclinical infection models¹⁰. However, DAL is not currently recommended for IE treatment.
64 Data evaluating the effect of DAL on a biofilm-producing infection and comparing its antimicrobial
65 activity to that of vancomycin in an original model could provide additional arguments for its
66 therapeutic use in the management of IE.

67 In addition, a model of this sort needs to highlight the bacterium-host-antibacterial interactions. Most
68 of the current IE models use *in vitro* or animal supports. These approaches are complex to implement
69 and raise ethical issues. We have therefore developed an *ex-vivo* model of human heart valve infection
70 by *S. aureus* so as to more easily study bacteria-valve interactions, including the role of biofilm in IE,
71 and also to consider bacterium-antibacterial interactions, in the presence of antibiotics.

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78 Material and methods

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80 Heart valve infection model

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82 Strains and conditions of bacterial growth

83 A clinical MRSA strain was isolated from blood cultures from a patient with proven health-care-
84 associated aortic IE. Genome sequencing was performed using the MinION® device (Oxford Nanopore
85 Technologies). Multilocus sequencing typing (MLST) and staphylococcal protein A (*spa*) typing were
86 obtained from genome sequencing.

87 Human aortic heart valves were obtained from non-infected patients undergoing valve replacement
88 surgery. Each heart valve collected was rinsed and cultured to ensure sample sterility. Freshly-removed
89 heart valves were infected according to a protocol derived from Avilés-Reyes *et al.*¹¹ Briefly, the
90 staphylococcal strain was grown in a brain-heart infusion broth (BHI, Oxoid®) at 37°C overnight. Non-
91 calcified valves areas were sectioned with biopsy punch in 8-millimeter diameter sections. The sections
92 were placed in 1mL of BHI containing a standardized inoculum at an optical density (OD) of 0.1,
93 corresponding to a final inoculum of $5 \cdot 10^7$ colony-forming units (CFU) per mL. The valve sections were
94 incubated for 48 hours at 37°C in brain-heart infusion broth (BHI, Oxoid®). The culture medium was
95 renewed at 24h. Each valve section was rinsed 3 times in phosphate buffer solution to remove poorly
96 adherent bacteria and thereafter sonicated with 500µL of Phosphate Buffer Saline (Sigma-Aldrich®) for
97 5 minutes (70kHz). The sonicated bacteria were plated to determine bacterial counts. The sterility of
98 each heart valve was assessed by plating an uninfected control after 48 hours in sterile BHI.

99 Biofilm formation

100 Initial bacterial adhesion to heart valve sections was primarily assessed using Gram staining performed
101 on paraffin-embedded histopathological sections of infected valve.

102 Biofilm formation was assessed by labelling infected heart valve cryosections with anti-poly-N- β -(1-6)-
103 acetyl-glucosamine (PNAG) antibody, i.e. an exopolysaccharide involved in the maturation stage of the
104 biofilm¹². PNAG antibody was coupled with a secondary antibody (Alexa Fluor 488TM, Thermofischer®
105 scientific) according to a protocol described by Maira-Litrán et al.¹³ The labelled valve sections were
106 analyzed by a fluorescence scanning system (NanoZoomer®, Hamamatsu). The presence of biofilm was
107 confirmed by surface analysis of sections of infected valves by scanning electron microscopy (SEM),
108 (Jeol®, Croissy Sur Seine, France). Valve sections previously infected and rinsed as described above
109 were dehydrated in ethanol followed by critical point drying assay and sputter coated with gold
110 palladium mixture.

111

112 **Antimicrobial susceptibility testing**

113

114 **MIC and MBC**

115 DAL and VAN were provided by Sigma Aldrich. The minimum inhibitory concentration (MIC) was
116 determined by the broth microdilution method according to CLSI guidelines for BHI broth with
117 standardized inocula. Samples exhibiting no visible bacterial growth were seeded and incubated
118 overnight. The minimum bactericidal concentration (MBC) was defined as the lowest concentration of
119 antibiotic that prevented 99.9% of the growth of bacteria.

120 **MbIC and MbEC**

121 The VAN and DAL Minimum biofilm inhibitory concentrations (MbIC) and minimum biofilm eradication
122 concentrations (MbEC) were determined by a modified Calgary device assay (called cMbIC/cMbEC)¹⁴,
123 by immersing pegs in a dedicated 96-well plate (MbEC Assay® Innovotech, Edmonton Canada)
124 containing 150 μ L of OD 0.1 adjusted inoculum for 24h at 37°C. After rinsing in sterile water, the peg
125 lids were incubated at 37°C for 24h in new wells containing 150 μ L of twofold dilutions of DAL (with
126 0.002% polysorbate 80 supplementation) or VAN in BHI broth. The lowest antibiotic concentration for

127 which there was no visible bacterial growth in well was identified as MbIC. Pegs exhibiting no visible
128 bacterial in corresponding wells were rinsed and placed in a new 96-well plate containing BHI broth
129 medium and then gently vortexed for 5 min at 70rpm for biofilm removal. MbEC was determined after
130 24h of incubation at 37°C.

131 MbIC and MbEC in heart valve sections

132 VAN and DAL MbIC and MbEC using heart valves sections (called vMbIC and vMbEC) were respectively
133 determined on 2 mm heart valve sections in a standard 96-well plate by incubation in the same
134 conditions as described above. Valve sections in wells exhibiting no visible bacterial growth were rinsed
135 in sterile water thereafter sonicated for 5 min (70kHz) in a new 96-well plate containing BHI broth.
136 vMbEC was determined after 24h of incubation at 37°C.

137 Finally, heart valves were analyzed 48h after the onset of infection using SEM by adding DAL (with
138 0.002% polysorbate 80 supplementation) and VAN respectively at vMbEC after 24 h of incubation of
139 the heart valve sections.

140 All experiments were performed at least in triplicate, the three most frequent identical results were
141 retained. The study was approved by the Ethics Committee of Rennes (registration number 19.92).

142 Statistical analysis

143 Statistical analysis was performed with Prism 5.0[®] software (GraphPad Software, La Jolla, CA, USA)

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151 Results

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153 Strain sequencing

154 Genome sequencing of staphylococcal isolates revealed clonal complex (CC) 8, sequence-type (ST) 8
155 *spa* type t008 strains, which was Panton-Valentine leucocidin negative. ST8 is the predominant *S.*
156 *aureus* clone in Rennes university hospital. Pattern of antimicrobial resistance genes is presented in
157 table S1.

158 Bacterial counts and biofilm formation

159 Bacterial counts on infected heart valves showed repeatable mean bacterial loads of 5.6 ± 6.10^7 CFU/mL
160 for the MRSA strain. Gram staining on histopathological sections of infected heart valves demonstrated
161 peripheral bacterial adherence on damaged areas as shown in fig. 1. Wide-field fluorescence analysis
162 of heart valve cryosections previously labelled with PNAG and its secondary fluorescent antibody
163 revealed specific areas of fluorescence, overlapping with the presence of staphylococcal colonies
164 revealed by the subsequent Gram staining (figS1).

165 SEM analysis performed on 48-hour infected heart valve sections confirmed the presence of numerous
166 colonies of aggregated staphylococci embedded in a matrix (fig.2), and confirmed the presence of a
167 biofilm on damaged valve areas.

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170 Susceptibility testing and biofilm assays

171 DAL and VAN MIC/MBC, cMbIC/cMbEC, vMbIC/ vMbEC are summarized in table 1.

172

Table 1. Dalbavancin and vancomycin activity assessed on planktonic (MIC/MBC) and sessile conditions (MbIC/ MbEC) on Calgary device and heart valve sections.

	<i>S. aureus</i> clinical strain					
	planktonic		Calgary device		heart valve	
	MIC	MBC	cMbIC	cMbEC	v MbIC	vMbEC
DAL	<0.01	<0.01	<0.01	0.06	<0.01	0.03
VAN	1	16	28	>64	28	32

MIC: minimum inhibitory concentration, MBC: minimum bactericidal concentration

cMbIC: minimum biofilm inhibitory concentration on Calgary device, MbEC: minimum biofilm eradication concentration on Calgary device

vMbIC: minimum biofilm inhibitory concentration on heart valves sections, MbEC: minimum biofilm eradication concentration on heart valves sections

Values are expressed in mg/L.

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175 SEM analysis of the treated heart valves showed reduced staphylococcal colonies at vMbEC for DAL

176 and VAN (see figure S2).

177

178 Discussion

179 IE remains a hard to treat infection. As the low prevalence of IE makes clinical studies difficult to
180 conduct, there is an urgent need for alternative models to improve patients management. Most of the
181 knowledge regarding biofilm development in infective endocarditis has been provided from pre-
182 clinical data, mainly on *in vitro* or animal models (rabbit and rat models)¹⁵. However, these models
183 raise the issue of transposability to humans and may not strictly control all the different experimental
184 conditions. Previous developments of *ex vivo* models of *Enterococcus faecalis* IE on a porcine valve
185 compared to biofilm formation on an abiotic substrate demonstrated the limited relevancy of *in vitro*
186 assays, given their ability to form biofilm on polystyrene for only half of the isolates, whereas all

187 isolates formed biofilm on the *ex vivo* model¹⁶. These findings strengthen the need for alternative
188 approaches, especially on human tissue to mimic human IE physiopathology in strictly controlled
189 experimental conditions¹⁷.

190 Herein, we have established adhesion followed by biofilm production on human heart valves infected
191 by *S. aureus*. The experimental conditions of our model are consistent with previous data indicating
192 that the extracellular matrix of staphylococcal biofilms is completely formed after 6 to 9h of incubation
193 ¹⁸. In addition, as staphylococcal IE is associated with early valve damage, **assessment of**
194 **antistaphylococcal activity on a model mimicking IE conditions** remains particularly challenging to
195 better evaluate the efficacy of treatments for staphylococcal IE.

196 Only retrospective data on IE patients treated with DAL is available, with cure rates ranging from 72
197 to 91%, but DAL was usually used after several lines of treatment which had resulted in prior
198 negatization of blood samples before the onset of DAL¹⁹. Although both VAN and DAL inhibit bacterial
199 cell wall synthesis, there are very few comparisons of their respective activity for IE management.
200 Previous *in vitro* studies of *S. aureus* biofilms have shown the superiority of DAL over VAN^{20,21}. DAL
201 has been compared in a single study to VAN in a rabbit model of staphylococcal IE and it showed
202 greater efficacy than VAN²⁰. Although our experimental conditions **do not include either** platelets and
203 fibrin, which are involved in the formation of vegetation, nor the effect of the immune response, our
204 results are consistent with this animal model, with a 28-fold increase in VAN MIC on a pre-established
205 biofilm. **Although our study is actually based on a single clinical isolate**, these values confirm the
206 interest of our easy-to-implement alternative model to study antibiofilm activity on human device.

207 Our results were also similar for both the Calgary device and our *ex-vivo* model. DAL showed strong
208 antibiofilm activity with cMbIC <0.01 mg/L. This value is consistent with documented MbIC₉₀ values of
209 <1 mg/L in a recent review¹⁰. DAL showed comparable cMbEC for pegs to that for heart valves
210 (0.03 and 0.06 mg/L respectively). Thus, DAL showed very encouraging results on human biological
211 device with strong activity, including on biofilm, and with very low vMbEC (0.03 mg/L). This value is

212 largely achieved in plasma concentrations measured in humans²². In addition to its potentially high
213 level of activity, DAL nephrotoxicity could be less frequent than it is with VAN²³. This characteristic
214 could be an additional argument for its use in IE where a staphylococcal etiology has been associated
215 with an increased risk of renal failure, leading to additional difficulties in the management with VAN
216 treatment²⁴. Taken together, our results on a human model confirm previous *in vitro* data and can
217 support the use of DAL as an alternative to VAN in IE treatment induction or as output relay.

218

219 Conclusion

220 Dalbavancin showed promising antibiofilm activity compared to vancomycin in an original *ex-vivo*
221 MRSA infective endocarditis model. Interestingly, dalbavancin remained highly bactericidal, including
222 on biofilm, with very low MIC and MbEC values. Our results provide new evidence of DAL antibiofilm
223 activity applied to IE, and this could provide further evidence for its potential in the medical
224 management of this pathology.

225

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228

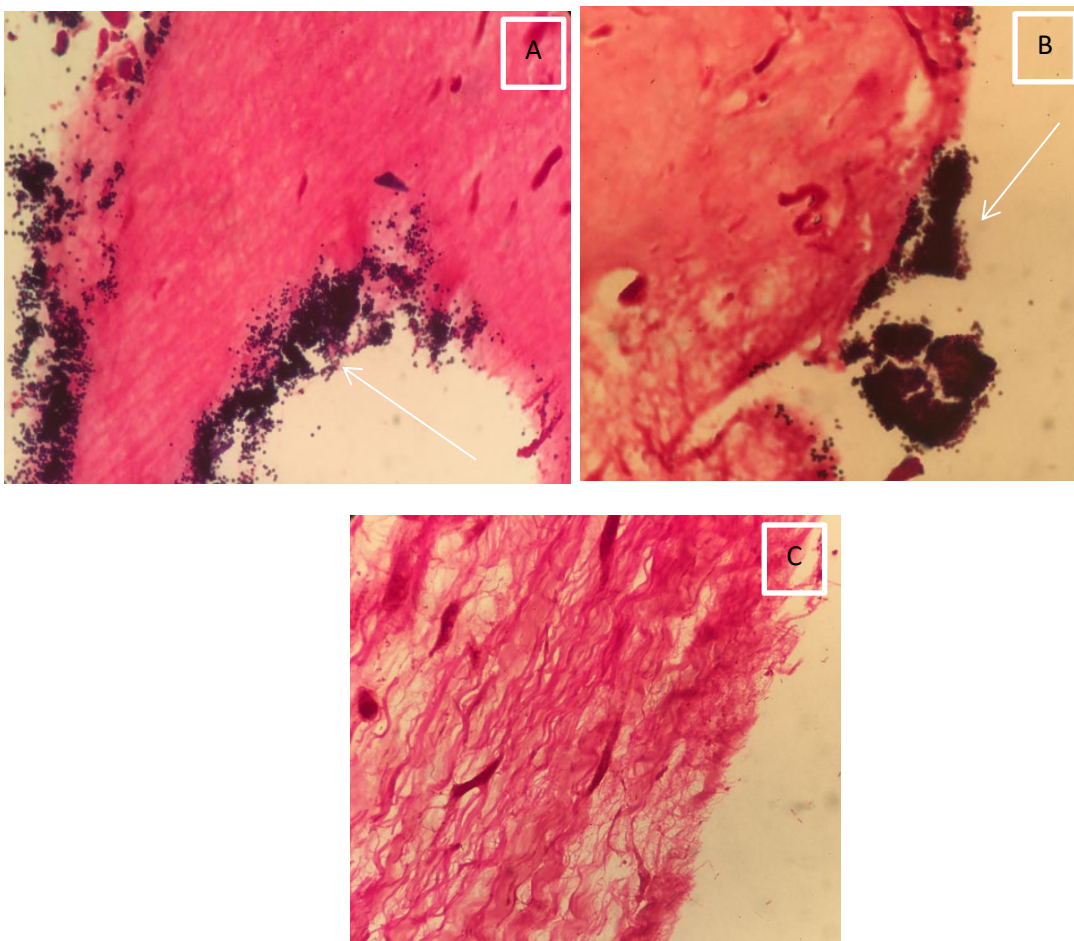
229 Transparency declarations

230 No conflicts of interest to report

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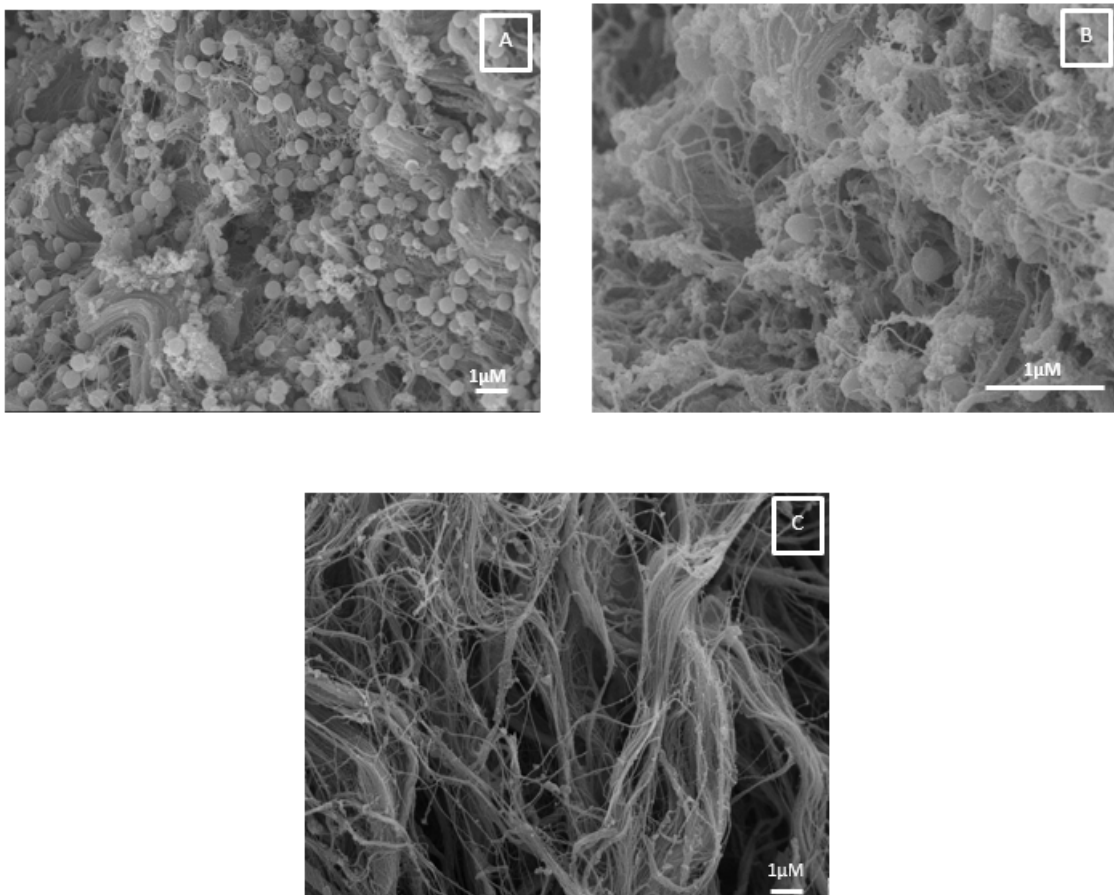
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247 **Figure 1.** Gram staining on histopathological sections of infected **(A, B)** and non-infected heart valves
248 **(C)**. Staphylococcal colonies (arrows) are marginally adherent to valves. (Magnification, x20).

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Figure 2. SEM images of 48h infected heart valves sections **(A)** (magnification, x5,000) with production of staphylococcal biofilm on heart valve structure **(B)** (magnification, x10,000) and non-infected heart valve section **(C)** (magnification, x5,000).

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