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1 Original research paper

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3 Title:

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5 Extended antimicrobial susceptibility assay for *Staphylococcus aureus*
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22
23
24 **Keywords:** Mastitis, susceptibility, biofilm, antimicrobials, *Staphylococcus aureus*,

25

26 **Abstract:**

27 *Staphylococcus aureus* is one of the most prevalent causes of bovine mastitis. The
28 antimicrobial treatment of this disease is currently based on antimicrobial susceptibility tests
29 according to CLSI standards. However, various studies have shown that there is a discrepancy
30 between the results of this standard susceptibility test and the actual cure rate of the applied
31 antimicrobial treatment. Increasing evidence suggests that biofilm formation by *S. aureus* is
32 associated with this problem. The currently available antimicrobial susceptibility assays for
33 bacteria growing in biofilms, are not considered reliable enough for routine application.
34 Therefore, the objective of this study was to further develop a susceptibility test for bacteria
35 growing in biofilm, suitable for routine testing of the antimicrobial susceptibility of *S. aureus*.
36 With the expansion of the available MBECTTM assay to an extended biofilm susceptibility test,
37 that comprises two and four consecutive days of antimicrobial challenge, the antimicrobial
38 susceptibility for *S. aureus* growing in biofilm was further analysed. The results showed clear
39 differences between strains and various antimicrobial agents with respect to the effect of
40 longer duration of the antimicrobial challenge on the eradication of *S. aureus* growing in
41 biofilm. The extended biofilm susceptibility test also indicates that each bacterial strain
42 requires a specific duration of antimicrobial therapy, which cannot be derived from a standard
43 susceptibility test or from a 24-hour biofilm susceptibility test.

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47

48 **Introduction.**

49 *Staphylococcus aureus* is one of the major causes of subclinical, clinical, recurrent and
50 chronic mastitis in dairy cattle. These infections are commonly treated with antimicrobial
51 agents and it is known, based on various epidemiological studies, that only a moderate
52 correlation exists between the antimicrobial susceptibility of *S. aureus in vitro* and its
53 bacteriological cure after antimicrobial therapy of the patient (Sol et al., 2000; Sol et al.,
54 1997). In fact treatment of cows suffering from chronic mastitis due to *S. aureus* infection
55 often fails completely, regardless of the antimicrobial susceptibility determined in a diagnostic
56 laboratory (Taponen et al., 2003; Wilson et al., 1999).

57 In human medicine, therapy- resistant, recurrent and chronic nosocomial infections caused by
58 staphylococci have been associated with the growth of these bacteria in biofilms (Dunne,
59 2002; Vuong and Otto, 2002).

60 Increasing evidence indicates that biofilm formation by *S. aureus* at the site of infection is
61 also the explanation for the apparent therapy resistance of *S. aureus* isolates causing bovine
62 mastitis (Cucarella et al., 2004; Melchior et al., 2006b; Melchior et al., 2006a; Oliveira et al.,
63 2006). It has been demonstrated that both *S. aureus* isolates obtained from bovine mastitis
64 and clinical *S. aureus* isolates from humans, are 10-1000 times more resistant to antimicrobial
65 agents when growing in biofilm, than the same isolate, growing in planktonic (free floating)
66 form (Amorena et al., 1999; Ceri et al., 1999; Melchior et al., 2006b; Olson et al., 2002).

67 Although several tests for the determination of the susceptibility to antimicrobials of bacteria
68 growing in biofilm, including the MBEC™ assay (Innovotech Inc¹, Edmonton, Canada), have
69 been developed, these assays are not yet considered reliable enough for routine application.

70 Furthermore, the results from a comparison of the susceptibility of *S. aureus* isolates from
71 bovine mastitis growing in biofilm against a broad range of antimicrobials (Melchior et al.,

¹ Formerly MBEC BioProducts Inc.

72 2006b) revealed surprisingly little difference between strains and antimicrobials. The 24h
73 biofilm susceptibility test resulted in all cases in a Minimal Biofilm Eradication Concentration
74 (MBEC) higher than the concentration that can be reached *in vivo*, indicating that all strains
75 were almost identically therapy resistant. This observation, together with the results obtained
76 during several clinical trials, which demonstrated that the chance of a positive therapy
77 outcome increases with a longer duration of the therapy (Pyorala and Pyorala, 1998; Sol et al.,
78 2000), led to the development of the extended MBEC assay.

79 In this extended assay, the MBEC is determined after two and four days of antimicrobial
80 challenge, whereas in the normal MBEC™ assay the MBEC concentration is determined after
81 24 hours of antimicrobial challenge (Ceri et al., 1999; Melchior et al., 2006b; Olson et al.,
82 2002). Furthermore, the Biofilm Minimal Inhibitory Concentrations (BMIC) were derived
83 with antimicrobial challenge plates from four consecutive days in the extended MBEC assay,
84 whereas in the normal MBEC™ assay the BMIC is determined once in a 24 h assay.

85 The aim of the present study was to compare the antimicrobial susceptibility of *S. aureus*
86 isolates obtained from bovine mastitis in the extended two and four day MBEC assay with
87 that of the one day MBEC assay. Secondly, it was evaluated whether the extended assay is
88 better suited for differentiation of the *in vitro* susceptibility of *S. aureus* strains growing in
89 biofilm. This would allow for a better comparison between strains and antimicrobials.

90 Four strains used in a previous study with the MBEC™ assay (Melchior et al., 2006b) were
91 tested in the extended MBEC biofilm susceptibility assay. Antimicrobials were selected based
92 on their usage and registration for the control of mastitis caused by *S. aureus*. The assays were
93 conducted both in CAMHB (Mueller Hinton Broth with cation adjustments according to the
94 CLSI guidelines, Sigma, St Louis, USA) and in Ultra Heated (UHT) milk.

95

96 **Materials and Methods**

97 **2.1 Bacterial strains and media**

98 The standard reference strain *S. aureus* Newbould 305 (ATCC 29740) (Prasad and
99 Newbould, 1968) and three field isolates from bovine mastitis (Hensen et al., 2000; Melchior
100 et al., 2006b) were used in this first study. Prior to the experiments, the strains were stored at
101 -70°C .

102 Strains Newbould 305, and BMA/GE/032/0412 (designated N305, and 0412 respectively) are
103 penicillin susceptible strains whereas strains BMA/UK/032/0106 and BMA/GE/032/0385
104 (designated 0106, and 0385) are penicillin resistant strains. All four strains are susceptible,
105 according to standard CLSI assays, for all other antimicrobials tested (Melchior et al., 2006b).
106 The growth media used in the biofilm assay (see below) were CAMHB and commercial UHT
107 milk with 3.5 % protein and 1.5 % fat. The UHT milk was buffered with 83 mmol HEPES (4-
108 (2-hydroxyethyl)-1-piperazineethanesulfonic acid), with the exception of UHT milk in which
109 strain 0412 was grown, where 200 mmol HEPES had to be used. The growth medium for all
110 antimicrobial challenge plates was CAMHB.

111 All inocula to be used in antimicrobial susceptibility tests were freshly prepared from
112 overnight sheep blood agar plates (Biotrading, Mijdrecht, Netherlands).

113

114 **2.2 Extended Antimicrobial susceptibility assay for bacteria growing in biofilms**

115 Measurements of the antimicrobial susceptibility of bacteria growing in biofilms were
116 performed with the MBEC™ biofilm assay. In brief, biofilms are allowed to form on the
117 surface of 96 pegs on the peg- lid of the assay. These biofilms were found to be statistically
118 equivalent (Ceri et al., 1999) and were subsequently exposed in 96- well plates for a variable
119 period of time to growth medium containing antimicrobials in different concentrations, to
120 allow determination of the susceptibility of the bacteria grown in the biofilm for these
121 antimicrobial agents (Ceri et al., 1999).

122 The assay was performed as previously described (Ceri et al., 1999; Melchior et al., 2006b)
123 with some modifications. In brief (see also Fig. 1), antimicrobial challenge was extended by
124 replacement of the antimicrobial challenge plates every 24 hours during two and four
125 consecutive days. During this period the 96-well plates were incubated at 37°C. BMIC
126 concentrations were determined from the challenge plates for two or four day periods,
127 respectively. MBEC concentrations were determined with the presence of visible bacteria
128 present after sonication for 5 min (Branson sonicator, Branson, Danbury, CR, USA) and 20 h
129 incubation at 37°C of the MBEC assay peg-lid in the CAMHB recovery plates.
130 The presence of visible growth, in both the BMIC and MBEC assay was determined by
131 measuring the optical density at 655nm in a 96-well plate reader (Biorad plate reader, Bio-rad,
132 Hercules, CA, USA).
133 The assays were performed in duplicate and in triplicate in two independent experiments, and
134 the results are presented as the mean antimicrobial dilution concentration from these five
135 assays. Quality controls were performed according to the MBEC protocols².
136 The concentrations of the antimicrobials used in the two and four day assays ranged between
137 0.5µg/mL and 512µg/mL for amoxicillin/ clavulanic acid (ratio 2:1), cefquinome,
138 cefoperazon, cloxacillin and pirlimycin, between 0.12µg/mL and 128µg/mL for penicillin and
139 penicillin/ neomycin (ratio 1:2) and between 0.25µg/mL and 256µg/mL for trimethoprim/
140 sulfamethoxazole (ratio 1:19). The antimicrobial concentrations ranges for the MBEC 1d
141 assay were different, and are indicated in the tables 1 and 2.

142

143 **3. Results**

144 **3.1 Extended Antimicrobial susceptibility assay for bacteria growing in biofilm**

² (http://www.innovotech.ca/products_instructions.php)

145 The results obtained in the extended MBEC assay are presented in Tables 1 and 2. In these
146 tables the MBEC values obtained after challenge with the selected antimicrobials for two and
147 four-days (MBEC 2d and MBEC 4d) are compared with the MBEC values obtained in the 24
148 h MBEC assay (MBEC 1d). The Minimal Inhibitory Concentrations (MIC) as defined in the
149 CLSI guidelines (NCCLS, 2002) and the MBEC 1d values have been published previously
150 (Melchior et al., 2006b). The Biofilm Minimal Inhibitory Concentrations (BMIC) was derived
151 from the extended incubations performed in this study.

152 Extended antimicrobial challenge for two and four-days of strain 0385 resulted in a slight
153 reduction of the MBEC concentrations, irrespective of whether CAMHB or UHT was used as
154 the growth medium for biofilm formation. Of all eight antimicrobials tested on CAMHB
155 grown biofilms, only cefquinome and pirlimycin resulted in at least 75% lower antimicrobial
156 concentrations necessary for biofilm eradication in the extended four-day challenge. The use
157 of trimethoprim-sulfamethoxazole in the extended four-day challenge resulted in an
158 antimicrobial concentration necessary for biofilm eradication that was at least 50% lower.
159 After four-day antimicrobial challenge of the biofilm formed by strain 0385 grown in UHT
160 medium, eradication of this biofilm could be obtained with only 1.6% of an antimicrobial
161 concentration of the antimicrobial pirlimycin that was *NOT* able to cause eradication after 24
162 hours of challenge.

163 Reference strain N305 showed the highest decrease in antimicrobial concentration necessary
164 for eradication after a four-day challenge as compared to a one-day challenge with
165 cefquinome, where only 3% of the 24-hour antimicrobial concentration was needed to obtain
166 biofilm eradication after four days. Extended challenge with penicillin decreased the
167 concentration of this antimicrobial needed for eradication of the biofilm by 94%. For all other
168 antimicrobials tested the concentrations were reduced to 12- 25% of the concentration needed
169 for eradication in the 24-hour assay. When UHT medium was used for growth of the biofilm,

170 the extended four-days challenge with amoxicillin/ clavulanic acid resulted in the largest
171 decrease observed; less than 1% of the two-day eradication concentration was already
172 effective when the biofilm was challenged for four days (MBEC d1 results not available).

173 For strain 0412, the MBEC 4d concentrations showed a sharp reduction when compared to the
174 MBEC 1d concentrations for the antimicrobials penicillin, cloxacillin and amoxicillin/
175 clavulanic acid. The MBEC 4d concentrations needed in these cases was less than 0.5% of the
176 MBEC 1d concentrations. The results for the extended challenge with cefquinome,
177 trimethoprim-sulfamethoxazole and neomycin/ penicillin indicated that only 1% of the MBEC
178 1d concentration was needed for eradication after four days of challenge. For all other
179 antimicrobials tested, little (pirlimycin) or no decrease in the concentration needed for biofilm
180 eradication was observed after an extended antimicrobial challenge. The results obtained for
181 biofilms grown in UHT medium were similar to those obtained after growth in CAMHB
182 medium, with the exception of those obtained with trimethoprim-sulfamethoxazole.

183 Strain 0106 is penicillin resistant and therefore, as can be expected, a longer challenge period
184 with this antimicrobial did not result in a decrease of MBEC values. However, the MBEC 4d
185 concentrations needed for cefquinome, pirlimycin and trimethoprim-sulfamethoxazole were
186 less than 0.2% of those needed for eradication of the biofilm in the MBEC 1d assay.

187 Extended challenge with all other antimicrobials tested during four days of challenge resulted
188 in a decrease of the concentration needed for biofilm eradication to only 1% of the MBEC 1d
189 concentration. With UHT as the growth medium for the biofilm, antimicrobial challenge with
190 cloxacillin, cefquinome, pirlimycin and trimethoprim-sulfamethoxazole during four days
191 resulted in a 97 - 99% reduction of the MBEC concentration needed after four days of
192 challenge in comparison with the concentration needed after a 24 hour challenge. The
193 antimicrobial MBEC concentration for neomycin/ penicillin and cefoperazon was reduced by
194 87 – 94 % in the four-day challenge, compared to that in the MBEC 1d challenge.

195

196 **4. Discussion and Conclusion**

197 The selection of antimicrobial agents for therapeutic use is generally based on susceptibility
198 testing of the isolated pathogens conducted according to the standards of the CLSI. In this
199 method the susceptibility of fast growing planktonic bacteria is measured.

200 Accumulating evidence indicates however, that growth of *S. aureus* in the bovine udder in the
201 form of a biofilm is the cause of the poor prediction of bacteriological cure when the data
202 obtained in the CLSI tests are followed (Melchior et al., 2006b; Melchior et al., 2006a; Olson
203 et al., 2002). The difficulty to produce reliable data for susceptibility of isolated field strains
204 implies that it is virtually impossible to choose the optimum antimicrobial agent and treatment
205 protocol under practical conditions. This results in numerous antimicrobial therapies without
206 the desired cure of bovine mastitis. The development of a reliable assay suitable for routine
207 testing, would improve the therapeutic intervention of bovine mastitis with antimicrobials
208 considerably. Furthermore, it would have important implications for dairy economics and
209 animal welfare.

210 Four field isolates of *S. aureus* from bovine mastitis were used in our experiments, all were
211 found susceptible to the antimicrobials tested, with exception of the penicillin resistance for
212 strain 0106 and 0385. It was shown that three of the strains showed a decrease in MBEC value
213 after the extended antimicrobial challenge. The exception was strain 0385, where the extended
214 challenge method did not, or only to a very limited extent yield a decrease in the minimal
215 biofilm eradication concentration. Since the MBEC 4d concentrations needed for strain 0385
216 were significantly higher than the antimicrobial concentrations that can be reached *in vivo*, we
217 consider this strain to behave as a true therapy resistant strain in this *in vitro* model.

218 The reference strain N305 in general revealed only moderate decreases in minimal biofilm
219 eradication concentrations after extended challenge, in comparison to both the second

220 penicillin susceptible strain 0412 and the penicillin resistant strain 0106. Strain N305 has been
221 used in several bovine mastitis studies (Hensen et al., 2000; Schukken et al., 1999), including
222 some studies on experimental mastitis infection and susceptibility (Owens, 1987; Owens et
223 al., 1993; Owens et al., 1993). From these studies it can be concluded that this strain is able to
224 cause clinical and mild chronic mastitis infections and has a consistent susceptibility for a
225 range of antimicrobials. The efficacy of the therapy after experimental infections with strain
226 N305 (Schukken et al., 1999) with two antimicrobials resulted in bacteriological cure in 30-
227 and 70% of the cases, depending on the antimicrobial used (Y.H. Schukken personal
228 communication).

229 For strains 0412 and 0106 a significant a significant decrease in the MBEC value was
230 observed in the extended antimicrobial challenge for several antimicrobials. However, there
231 are significant differences (i.e. for cloxacillin and cefquinome) between some of the tested
232 antimicrobials. A decrease of at least 99% in MBEC concentrations for all β -lactam
233 antimicrobials, with the exception of cefoperazon, was observed for strain 0412. Furthermore,
234 after four days of antimicrobial challenge, the MBEC concentrations for cloxacillin and
235 amoxicillin/ clavulanic acid were within the susceptibility range according to the CLSI
236 guidelines. Although these guidelines do not apply for biofilm susceptibility assays, it can be
237 assumed that these concentrations can be reached *in vivo*. For strain 0106, a penicillin
238 resistant strain, a decrease of 99 – 99,8% in MBEC concentrations for cefquinome,
239 cloxacillin, pirlimycin and trimethoprim-sulfamethoxazole was observed.

240 Most of the tested antimicrobials have a time dependent activity, however, no linear
241 correlation between MBEC 1d values and MBEC 4d outcomes could be established, mainly
242 because of large differences observed in the decrease of the MBEC values after extended
243 challenge. In two reports time dependent β -lactam antimicrobials were tested in a clinical trial
244 with treatment regimens of different length (Oliver et al., 2004; Sol et al., 2000). In both

245 trials bacteriological cure rates were increased upon longer duration of therapy, however Sol
246 and coworkers observed a significant difference between penicillin susceptible and penicillin
247 resistant strains in this respect. The differentiation of *S. aureus* strains according to their
248 penicillin susceptibility, showed that an increased bacterial cure rate from extended therapy is
249 very limited in the case of penicillin resistant strains.

250 A comparison of the BMIC and MBEC concentrations determined in CAMHB and UHT-
251 milk shows that each strain reacts different on this change of growth medium. Therefore it
252 was not possible to reveal a specific effect of these growth media on the biofilm antimicrobial
253 susceptibility for any of the antimicrobials tested. Although the MBEC d1 concentrations are
254 generally lower for biofilms grown in UHT milk than for biofilms grown in CAMHB, the
255 values obtained in the extended assay for bacterial biofilms grown in UHT and CAMHB show
256 that the majority (90%) of the obtained results are in the same range for both the MBEC d2
257 and MBEC d4 assay. Previous studies showed that *S. aureus* bacterial biofilms grown in milk
258 medium contain inclusions of milk-fat and- protein particles, and are less compact compared
259 to biofilm grown in standard media (Amorena et al., 1999). The presence of fat and protein
260 particles in the biofilm might explain differences in the susceptibility to individual
261 antimicrobials with a similar mechanism of action, as in this case, the efficacy is also
262 influenced by physico-chemical characteristics of the antimicrobial.

263 However, apparently strain specific effects have more influence on the results in this assay.
264 For example strain 0106 showed a lower susceptibility towards for cloxacillin, and
265 amoxicillin/ clavulanic acid when grown in milk, whereas strain N305 was found to be more
266 susceptible for these antimicrobials if biofilms are grown in UHT. These latter results indicate
267 that the observed variability is strain-dependent and not antimicrobial-dependent.

268 The marked differences observed in the decrease of MBEC values after extended challenge
269 suggest, that each strain requires a strain specific time period during which antimicrobial

270 treatment should be maintained, independent of the individual MIC, BMIC or MBEC 1d
271 values. Although *in vivo* circumstances will be different for each specific case, with respect to
272 bacterial load and environmental growth conditions, the extended challenge method might
273 provide a tool for the evaluation of the duration of antimicrobial therapy required for an
274 adequate therapy result.

275 Whether these biofilm susceptibility differences observed *in vitro* are caused by genetic,
276 metabolic, regulatory or other mechanisms remains to be investigated.

277 In conclusion, the presented model of an extended MBEC assay in which the susceptibility of
278 *S. aureus* biofilm against a panel of common antibiotics was tested, indicates marked
279 differences when these results were compared with common standard procedures such as the
280 CLSI method for planktonic bacteria, or the commercial MBEC™ assay. The extended
281 MBEC assay seems to correlate with the principle of time-dependent effects of the selected
282 antimicrobials as the MBEC concentrations decreased considerably with time, albeit in a
283 strain-dependent manner. At present only four *S. aureus* strains, isolated from cases of bovine
284 mastitis could be tested. The obtained results warrant further investigations with a larger
285 number of field isolates, as this extended MBEC assay might be developed into a valuable
286 tool to predict the outcome of a prolonged therapy under *in vivo* conditions.

287

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358 mastitis pathogens. *J. Dairy. Sci.* 82, 1664-1670.
- 359
- 360

361 Table 1

CAMHB	Method	Conc range (µg/ml)	strains			
AB			N305	0412	0106*	0385*
Pen	MIC		≤0.06	≤0.06	4	>8
	BMIC	0.12-128	≤0.5	≤0.5	>128	>128
	MBEC 1d	2-1024	256	256	≥2048	≥2048
	MBEC 2d		64	64	>128	>128
	MBEC 4d	0.12-128	16	0.5	>128	>128
Clo	MIC		0.25	0.12	0.25	0.12
	BMIC	0.5-512	≤0.5	≤0.5	≤0.5	≤0.5
	MBEC 1d	2-1024	512	512	1024	1024
	MBEC 2d		256	256	32	512
	MBEC 4d	0.5-512	128	1	8	>512
Aug 2:1	MIC		≤0.5/0.25	≤0.5/0.25	≤0.5/0.25	1/0.5
	BMIC	0.5-512	≤0.5/0.25	≤0.5/0.25	1/0.5	4/2
	MBEC 1d	NA	NA	NA	NA	NA
	MBEC 2d		256/128	512/128	32/16	>512/256
	MBEC 4d	0.5-512	64/32	2/1	1/0.5	>512/256
Ceq	MIC		0.5	0.5	0.5	1
	BMIC	0.5-512	≤0.5	≤0.5	1	1
	MBEC 1d	2-1024	≥2048	1024	≥2048	≥2048
	MBEC 2d		256	512	64	>512
	MBEC 4d	512-0.5	64	16	1	512
Cfp	MIC		2	2	2	4
	BMIC	0.5-512	2	≤0.5	2	8
	MBEC 1d	2-1024	≥2048	256	≥2048	≥2048
	MBEC 2d		512	512	32	>512
	MBEC 4d	0.5-512	256	256	32	>512
Pirl	MIC		0.5	0.5	0.5	0.5
	BMIC	0.5-512	≤0.5	≤0.5	≤0.5	≤0.5
	MBEC 1d	2-1024	1024	512	≥2048	≥2048
	MBEC 2d	0.5-512	>512	512	64	512
	MBEC 4d		256	64	4	512
SXT 1:19	MIC		≤0.12	≤0.12	≤0.12	≤0.12
	BMIC	0.25-256	≤0.25	≤0.25	≤0.25	≤0.25
	MBEC 1d	2-1024	256	512	256	512
	MBEC 2d		128	256	4	>256
	MBEC 4d	0.25-256	64	8	0.5	256
Neo/pen 1:2	MIC		≤0.02/0.03	≤0.02/0.03	≤0.02/0.03	≤0.02/0.03
	BMIC	0.12-128	≤0.12/0.06	≤0.12/0.06	2/4	2/4
	MBEC 1d	0.5-512	32/64	64/128	≥512/1024	256/512
	MBEC 2d		64/128	16/32	16/32	128/256
	MBEC 4d	0.12-128	4/8	0.25/0.5	8/16	128/256

362 MIC, minimal inhibitory concentration; BMIC, Biofilm MIC; MBEC, minimal biofilm
 363 eradication concentration; AB, antimicrobial; Pen, penicillin; Clo, cloxacillin; Aug,
 364 amoxicillin/clavulanic acid; Ceq, cefquinome; Cfp, cefoperazon; Pirl, pirlimycin; SXT,
 365 trimethoprim sulphamethoxazole; Neo, neomycin.

366 *Strain N305 and 0412 are penicillin susceptible, strain 0106 and 0385 are penicillin resistant

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367 Table 2.

UHT	Method	Conc range (µg/ml)	strains			
AB			N305	0412	0106	0385
Pen	BMIC	0.12-128	<=0.12	<=0.12	256	32
	MBEC 1d	2-1024	128	128	>=2048	>=2048
	MBEC 2d	0.12-128	64	32	>128	>128
	MBEC 4d		16	0.5	>128	>128
Clo	BMIC	0.5-512	<=0.5	<=0.5	<=0.5	<=0.5
	MBEC 1d	2-1024	256	1024	1024	1024
	MBEC 2d	0.5-512	512	512	64	512
	MBEC 4d		32	1	32	256
Aug 2:1	BMIC	0.5-512	<=0.5/0.25	<=0.50.25	1/0.5	2/1
	MBEC 1d	NA	NA	NA	NA	NA
	MBEC 2d	0.5-512	256/128	512/256	64/32	>512/256
	MBEC 4d		2/1	2/1	32/16	512/256
Ceq	BMIC	0.5-512	<=0.5	<=0.5	1	1
	MBEC 1d	2-1024	>=2048	512	>=2048	>=2048
	MBEC 2d	0.5-512	>512	128	128	>512
	MBEC 4d		256	32	32	>512
Cfp	BMIC	0.5-512	<=0.5	<=0.5	4	8
	MBEC 1d	2-1024	>=2048	256	>=2048	>=2048
	MBEC 2d	0.5-512	>512	256	256	>512
	MBEC 4d		128	128	256	>512
Pirl	BMIC	0.5-512	<=2	<=0.5	<=0.5	<=0.5
	MBEC 1d	2-1024	256	1024	1024	>=2048
	MBEC 2d	0.5-512	512	256	32	512
	MBEC 4d		256	128	8	32
SXT 1:19	BMIC	0.25-256	<=0.25	<=0.25	<=0.25	<=0.25
	MBEC 1d	2-1024	128	8	256	16
	MBEC 2d	0.25-256	128	128	4	256
	MBEC 4d		64	128	2	64
Neo/pen 1:2	BMIC	0.12-128	<=0.12/0.25	<=0.12/0.25	0.5/1	1/2
	MBEC 1d	0.5-512	64/128	16/32	256/512	16/32
	MBEC 2d	0.12-128	64/128	8/16	32/64	128/256
	MBEC 4d		8/16	4/8	16/32	64/128

368 Explanation of abbreviations: see table 1.

369

370

371

372 Table 1. MIC, BMIC, MBEC 1d, MBEC 2d and MBEC 4d concentrations for strains N305,
373 0412, 0106 and 0385 for biofilms grown in CAMHB.

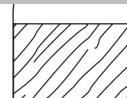
374

375 Table 2 MIC, BMIC, MBEC 1d, MBEC 2d and MBEC 4d concentrations for strains N305,
376 0412, 0106 and 0385 for biofilms grown in UHT milk.

377

378 Fig. 1. Flow diagram for extended antimicrobial challenge for biofilms. Aliquoutes of the same
379 inoculum are divided over separate MBEC assay plates for 24 h growth on a rocking
380 table at 37°C to facilitate the formation of a bacterial biofilm on the 96-pegs on the lid
381 of the MBEC assay plates. After 24h the peg-lid is mounted onto a 96-wells challenge
382 plate containing serial dilutions of the selected antimicrobials. The antimicrobial
383 challenge plate is replaced every 24 h by a new plate, i.e. once in the MBEC 2d assay
384 and three times in the MBEC 4d assay. The biofilm peg-lid and the antimicrobial
385 challenge plates are left for growth at 37°C. After two or four days of challenge, the
386 peg-lid is mounted in a 96-wells recovery plate with CAMHB (200µl) in each well.
387 After sonication, these plates are incubated at 37°C for 24 h. In all plates, bacterial
388 growth is measured as optical density in a 96 well plate reader.

Standard

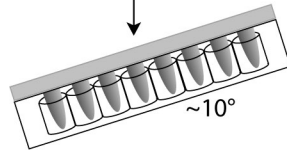
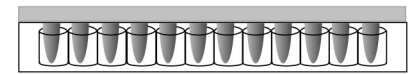
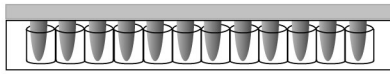
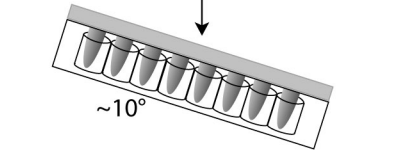
 $\sim 10^7$ cfu/ml

CAMHB

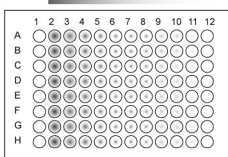
inoculate in duplicate

2 days MBEC Assay

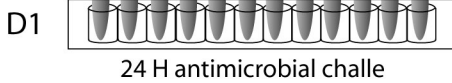
4 days MBEC Assay

 $\sim 10^\circ$  $\sim 10^\circ$

serial dilutions

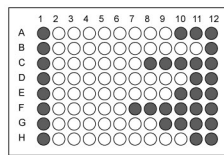
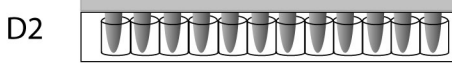
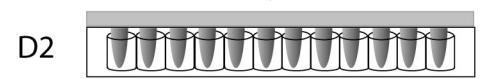


antimicrobial challenge plate

inoculate for 24 h
on rocking tableInsert peg lid into
antimicrobial challenge plateD1
24 H antimicrobial challenge

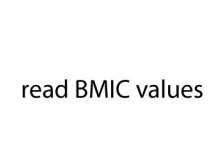
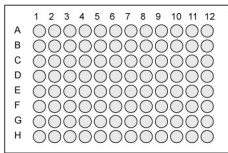
D1

24 H antimicrobial challenge

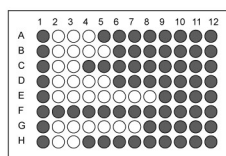
read BMC
valuesread BMC
valuesD2
2 X 24 H antimicrobial challenge

D2

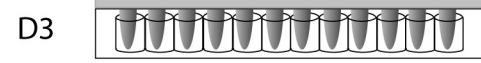
2 x 24 H antimicrobial challenge

read BMC
valuesread BMC
valuesCAMHB 200 μ l/well

recovery plate

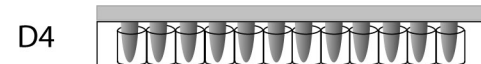
Load peg lid into recovery plate
and sonicate
24 H growth at 37°

read recovery



D3

3 x 24 H antimicrobial challenge

read BMC
values

D4

4 x 24 H antimicrobial challenge

read BMC
values