

Extended antimicrobial susceptibility assay for *Staphylococcus aureus* isolates from bovine mastitis growing in biofilms

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Abstract

Staphylococcus aureus is one of the most prevalent causes of bovine mastitis. The antimicrobial treatment of this disease is currently based on antimicrobial susceptibility tests according to CLSI standards. However, various studies have shown that there is a discrepancy between the results of this standard susceptibility test and the actual cure rate of the applied antimicrobial treatment. Increasing evidence suggests that biofilm formation by *S. aureus* is associated with this problem. The currently available antimicrobial susceptibility assays for bacteria growing in biofilms, are not considered reliable enough for routine application. Therefore, the objective of this study was to further develop a susceptibility test for bacteria growing in biofilm, suitable for routine testing of the antimicrobial susceptibility of *S. aureus*. With the expansion of the available MBECTM assay to an extended biofilm susceptibility test, that comprises 2 and 4 consecutive days of antimicrobial challenge, the antimicrobial susceptibility for *S. aureus* growing in biofilm was further analysed. The results showed clear differences between strains and various antimicrobial agents with respect to the effect of longer duration of the antimicrobial challenge on the eradication of *S. aureus* growing in biofilm. The extended biofilm susceptibility test also indicates that each bacterial strain requires a specific duration of antimicrobial therapy, which cannot be derived from a standard susceptibility test or from a 24-h biofilm susceptibility test.

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1. Introduction

Staphylococcus aureus is one of the major causes of subclinical, clinical, recurrent and chronic mastitis in dairy cattle. These infections are commonly treated with antimicrobial agents and it is known, based on various epidemiological studies, that only a moderate

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correlation exists between the antimicrobial susceptibility of *S. aureus in vitro* and its bacteriological cure after antimicrobial therapy of the patient (Sol et al., 1997, 2000). In fact, treatment of cows suffering from chronic mastitis due to *S. aureus* infection often fails completely, regardless of the antimicrobial susceptibility determined in a diagnostic laboratory (Taponen et al., 2003; Wilson et al., 1999).

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

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

Although several tests for the determination of the susceptibility to antimicrobials of bacteria growing in biofilm, including the MBECTM assay (Innovotech Inc.,¹ Edmonton, Canada), have been developed, these assays are not yet considered reliable enough for routine application. Furthermore, the results from a comparison of the susceptibility of *S. aureus* isolates from bovine mastitis growing in biofilm against a broad range of antimicrobials (Melchior et al., 2006b) revealed surprisingly little difference between strains and antimicrobials. The 24 h biofilm susceptibility test resulted in all cases in a Minimal Biofilm Eradication Concentration (MBEC) higher than the concentration that can be reached *in vivo*, indicating that all strains were almost identically therapy resistant. This observation, together with the results obtained during several clinical trials, which demonstrated that the chance of a positive therapy outcome increases with a longer duration of the therapy (Pyorala and Pyorala, 1998; Sol et al., 2000), led to the development of the extended MBEC assay.

In this extended assay, the MBEC is determined after 2 and 4 days of antimicrobial challenge, whereas in the normal MBECTM assay the MBEC concentration is determined after 24 h of antimicrobial challenge (Ceri et al., 1999; Melchior et al., 2006b; Olson et al., 2002). Furthermore, the Biofilm Minimal Inhibitory Concentrations (BMIC) were derived with antimicrobial challenge plates from 4 consecutive days in the extended MBEC assay, whereas in the normal MBECTM assay the BMIC is determined once in a 24 h assay.

The aim of the present study was to compare the antimicrobial susceptibility of *S. aureus* isolates obtained from bovine mastitis in the extended 2- and 4-day MBEC assay with that of the 1-day MBEC assay. Secondly, it was evaluated whether the extended assay is better suited for differentiation of the *in vitro* susceptibility of *S. aureus* strains growing in biofilm. This would allow for a better comparison between strains and antimicrobials.

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

2. Materials and methods

2.1. Bacterial strains and media

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

Strains Newbould 305, and BMA/GE/032/0412 (designated N305, and 0412, respectively) are penicillin susceptible strains whereas strains BMA/UK/032/0106 and BMA/GE/032/0385 (designated 0106, and 0385) are penicillin resistant strains. All four strains are susceptible, according to standard CLSI assays, for all other antimicrobials tested (Melchior et al., 2006b).

¹ Formerly MBEC BioProducts Inc.

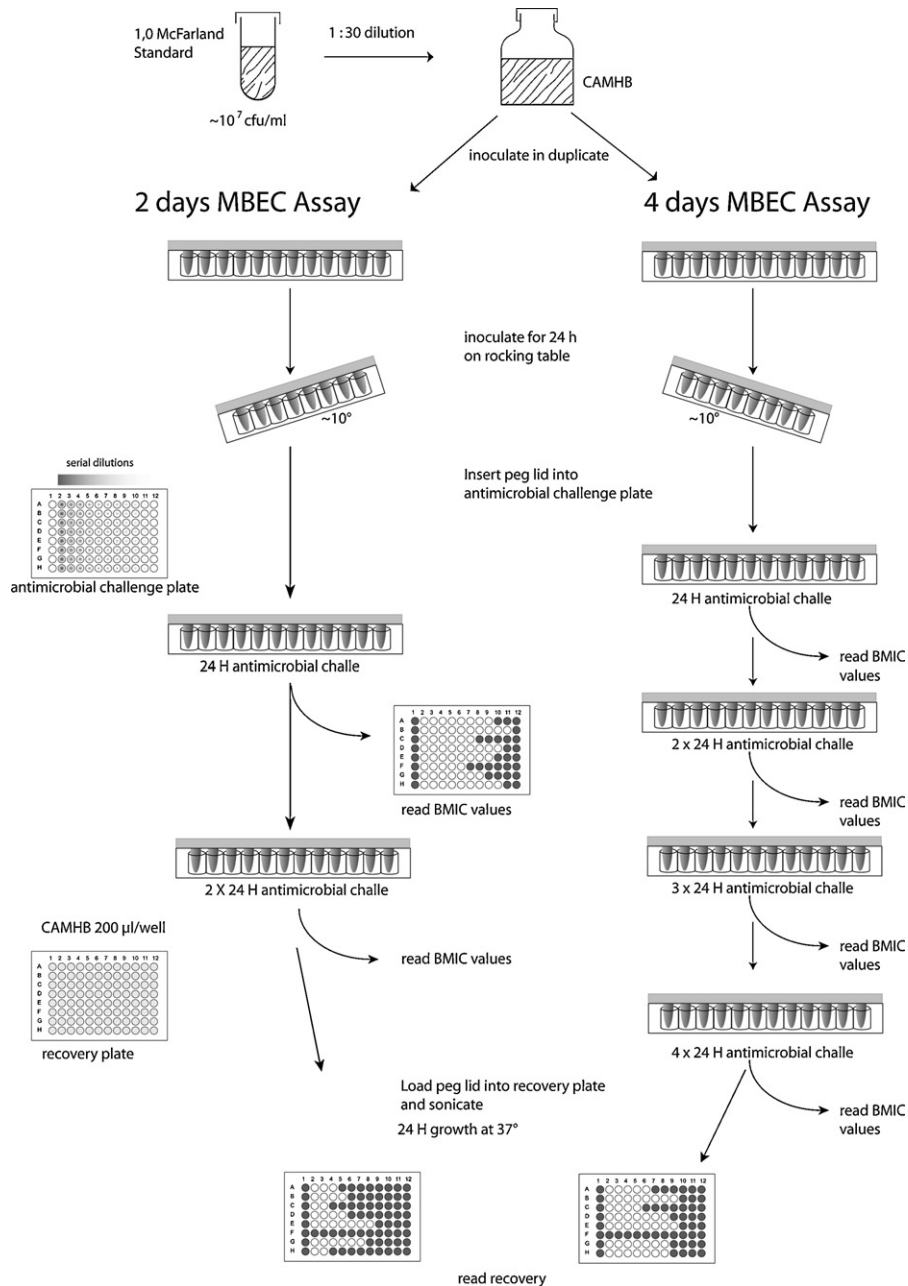


Fig. 1. Flow diagram for extended antimicrobial challenge for biofilms. Aliquotes of the same inoculum are divided over separate MBEC assay plates for 24 h growth on a rocking table at 37 °C to facilitate the formation of a bacterial biofilm on the 96-pegs on the lid of the MBEC assay plates. After 24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

Table 1

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

CAMHB (AB)	Method	Conc. range (µg/mL)	Strains			
			N305	0412	0106 ^a	0385 ^a
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	0.12–128	64	64	>128	>128
	MBEC 4d		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	0.5–512	256	256	32	512
	MBEC 4d		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	0.5–512	256/128	512/128	32/16	>512/256
	MBEC 4d		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	512–0.5	256	512	64	>512
	MBEC 4d		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	0.5–512	512	512	32	>512
	MBEC 4d		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	0.25–256	128	256	4	>256
	MBEC 4d		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	0.12–128	64/128	16/32	16/32	128/256
	MBEC 4d		4/8	0.25/0.5	8/16	128/256

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

^a Strain N305 and 0412 are penicillin susceptible, strain 0106 and 0385 are penicillin resistant

The growth media used in the biofilm assay (see below) were CAMHB and commercial UHT milk with 3.5% protein and 1.5% fat. The UHT milk was buffered with 83 mmol HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), with the exception of UHT milk in which strain 0412 was grown, where 200 mmol HEPES had to be used. The growth medium for all antimicrobial challenge plates was CAMHB.

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

2.2. Extended antimicrobial susceptibility assay for bacteria growing in biofilms

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

Table 2

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

UHT (AB)	Method	Conc. range (µg/mL)	Strains			
			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

Explanation of abbreviations: see Table 1.

determination of the susceptibility of the bacteria grown in the biofilm for these antimicrobial agents (Ceri et al., 1999).

The assay was performed as previously described (Ceri et al., 1999; Melchior et al., 2006b) with some modifications. In brief (see also Fig. 1), antimicrobial challenge was extended by replacement of the antimicrobial challenge plates every 24 h during 2 and 4 consecutive days. During this period the 96-well plates were incubated at 37 °C. BMIC concentrations were determined from the challenge plates for 2- or 4-day periods, respectively. MBEC concentrations were determined with the presence of visible bacteria present after sonication for 5 min (Branson Sonicator, Branson, Danbury, CR, USA) and 20 h incubation at 37 °C of the MBEC assay peg-lid in the CAMHB recovery plates.

The presence of visible growth, in both the BMIC and MBEC assay was determined by measuring the optical density at 655 nm in a 96-well plate reader (Biorad Plate Reader, Bio-Rad, Hercules, CA, USA).

The assays were performed in duplicate and in triplicate in two independent experiments, and the results are presented as the mean antimicrobial dilution concentration from these five assays. Quality controls were performed according to the MBEC protocols.²

The concentrations of the antimicrobials used in the 2- and 4-day assays ranged between 0.5 and 512 µg/mL for amoxicillin/clavulanic acid (ratio 2:1), cefquinome, cefoperazon, cloxacillin and pirlimycin, between 0.12 and 128 µg/mL for penicillin and penicillin/neomycin (ratio 1:2) and between 0.25 and 256 µg/mL for trimethoprim/sulfamethoxazole (ratio 1:19). The antimicrobial concentrations ranges for the MBEC 1d assay were different, and are indicated in Tables 1 and 2.

3. Results

3.1. Extended antimicrobial susceptibility assay for bacteria growing in biofilm

The results obtained in the extended MBEC assay are presented in Tables 1 and 2. In these tables, the MBEC values obtained after challenge with the selected antimicrobials for 2- and 4-days (MBEC

2d and MBEC 4d) are compared with the MBEC values obtained in the 24 h MBEC assay (MBEC 1d). The Minimal Inhibitory Concentrations (MIC) as defined in the CLSI guidelines (NCCLS, 2002) and the MBEC 1d values have been published previously (Melchior et al., 2006b). The Biofilm Minimal Inhibitory Concentrations was derived from the extended incubations performed in this study.

Extended antimicrobial challenge for 2- and 4-days of strain 0385 resulted in a slight reduction of the MBEC concentrations, irrespective of whether CAMHB or UHT was used as the growth medium for biofilm formation. Of all eight antimicrobials tested on CAMHB grown biofilms, only cefquinome and pirlimycin resulted in at least 75% lower antimicrobial concentrations necessary for biofilm eradication in the extended 4-day challenge. The use of trimethoprim-sulfamethoxazole in the extended 4-day challenge resulted in an antimicrobial concentration necessary for biofilm eradication that was at least 50% lower. After 4-day antimicrobial challenge of the biofilm formed by strain 0385 grown in UHT medium, eradication of this biofilm could be obtained with only 1.6% of an antimicrobial concentration of the antimicrobial pirlimycin that was *NOT* able to cause eradication after 24 h of challenge.

Reference strain N305 showed the highest decrease in antimicrobial concentration necessary for eradication after a 4-day challenge as compared to a 1-day challenge with cefquinome, where only 3% of the 24-h antimicrobial concentration was needed to obtain biofilm eradication after 4 days. Extended challenge with penicillin decreased the concentration of this antimicrobial needed for eradication of the biofilm by 94%. For all other antimicrobials tested the concentrations were reduced to 12–25% of the concentration needed for eradication in the 24-h assay. When UHT medium was used for growth of the biofilm, the extended 4-days challenge with amoxycillin/clavulanic acid resulted in the largest decrease observed; less than 1% of the 2-day eradication concentration was already effective when the biofilm was challenged for 4 days (MBEC d1 results not available).

For strain 0412, the MBEC 4d concentrations showed a sharp reduction when compared to the MBEC 1d concentrations for the antimicrobials penicillin, cloxacillin and amoxycillin/clavulanic acid. The MBEC 4d concentrations needed in these

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

cases was less than 0.5% of the MBEC 1d concentrations. The results for the extended challenge with cefquinome, trimethoprim-sulfamethoxazole and neomycin/penicillin indicated that only 1% of the MBEC 1d concentration was needed for eradication after 4 days of challenge. For all other antimicrobials tested, little (pirlimycin) or no decrease in the concentration needed for biofilm eradication was observed after an extended antimicrobial challenge. The results obtained for biofilms grown in UHT medium were similar to those obtained after growth in CAMHB medium, with the exception of those obtained with trimethoprim-sulfamethoxazole.

Strain 0106 is penicillin resistant and therefore, as can be expected, a longer challenge period with this antimicrobial did not result in a decrease of MBEC values. However, the MBEC 4d concentrations needed for cefquinome, pirlimycin and trimethoprim-sulfamethoxazole were less than 0.2% of those needed for eradication of the biofilm in the MBEC 1d assay. Extended challenge with all other antimicrobials tested during 4 days of challenge resulted in a decrease of the concentration needed for biofilm eradication to only 1% of the MBEC 1d concentration. With UHT as the growth medium for the biofilm, antimicrobial challenge with cloxacillin, cefquinome, pirlimycin and trimethoprim-sulfamethoxazole during 4 days resulted in a 97–99% reduction of the MBEC concentration needed after 4 days of challenge in comparison with the concentration needed after a 24 h challenge. The antimicrobial MBEC concentration for neomycin/penicillin and cefoperazone was reduced by 87–94% in the 4-day challenge, compared to that in the MBEC 1d challenge.

4. Discussion and conclusion

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

Accumulating evidence indicates however, that growth of *S. aureus* in the bovine udder in the form of a biofilm is the cause of the poor prediction of bacteriological cure when the data obtained in the CLSI tests are followed (Melchior et al., 2006a,b;

Olson et al., 2002). The difficulty to produce reliable data for susceptibility of isolated field strains implies that it is virtually impossible to choose the optimum antimicrobial agent and treatment protocol under practical conditions. This results in numerous antimicrobial therapies without the desired cure of bovine mastitis. The development of a reliable assay suitable for routine testing, would improve the therapeutic intervention of bovine mastitis with antimicrobials considerably. Furthermore, it would have important implications for dairy economics and animal welfare.

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

The reference strain N305 in general revealed only moderate decreases in minimal biofilm eradication concentrations after extended challenge, in comparison to both the second penicillin susceptible strain 0412 and the penicillin resistant strain 0106. Strain N305 has been used in several bovine mastitis studies (Hensen et al., 2000a,b; Schukken et al., 1999), including some studies on experimental mastitis infection and susceptibility (Owens, 1987; Owens et al., 1993a,b). From these studies it can be concluded that this strain is able to cause clinical and mild chronic mastitis infections and has a consistent susceptibility for a range of antimicrobials. The efficacy of the therapy after experimental infections with strain N305 (Schukken et al., 1999) with two antimicrobials resulted in bacteriological cure in 30 and 70% of the cases, depending on the antimicrobial used (Schukken, personal communication).

For strains 0412 and 0106 a significant decrease in the MBEC value was observed in the extended antimicrobial challenge for several antimicrobials. However, there are significant differences (i.e. for

cloxacillin and cefquinome) between some of the tested antimicrobials. A decrease of at least 99% in MBEC concentrations for all β -lactam antimicrobials, with the exception of cefoperazon, was observed for strain 0412. Furthermore, after 4 days of antimicrobial challenge, the MBEC concentrations for cloxacillin and amoxycillin/clavulanic acid were within the susceptibility range according to the CLSI guidelines. Although these guidelines do not apply for biofilm susceptibility assays, it can be assumed that these concentrations can be reached *in vivo*. For strain 0106, a penicillin resistant strain, a decrease of 99–99.8% in MBEC concentrations for cefquinome, cloxacillin, pirlimycin and trimethoprim-sulfamethoxazole was observed.

Most of the tested antimicrobials have a time-dependent activity, however, no linear correlation between MBEC 1d values and MBEC 4d outcomes could be established, mainly because of large differences observed in the decrease of the MBEC values after extended challenge. In two reports time-dependent β -lactam antimicrobials were tested in a clinical trial with treatment regimens of different length (Oliver et al., 2004; Sol et al., 2000). In both trials bacteriological cure rates were increased upon longer duration of therapy, however Sol et al. observed a significant difference between penicillin susceptible and penicillin resistant strains in this respect. The differentiation of *S. aureus* strains according to their penicillin susceptibility, showed that an increased bacterial cure rate from extended therapy is very limited in the case of penicillin resistant strains.

A comparison of the BMIC and MBEC concentrations determined in CAMHB and UHT-milk shows that each strain reacts different on this change of growth medium. Therefore, it was not possible to reveal a specific effect of these growth media on the biofilm antimicrobial susceptibility for any of the antimicrobials tested. Although the MBEC d1 concentrations are generally lower for biofilms grown in UHT milk than for biofilms grown in CAMHB, the values obtained in the extended assay for bacterial biofilms grown in UHT and CAMHB show that the majority (90%) of the obtained results are in the same range for both the MBEC d2 and MBEC d4 assay. Previous studies showed that *S. aureus* bacterial biofilms grown in milk medium contain inclusions of milk-fat and -protein particles, and are less compact

compared to biofilm grown in standard media (Amorena et al., 1999). The presence of fat and protein particles in the biofilm might explain differences in the susceptibility to individual antimicrobials with a similar mechanism of action, as in this case, the efficacy is also influenced by physico-chemical characteristics of the antimicrobial.

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

The marked differences observed in the decrease of MBEC values after extended challenge suggest, that each strain requires a strain specific time period during which antimicrobial treatment should be maintained, independent of the individual MIC, BMIC or MBEC 1d values. Although *in vivo* circumstances will be different for each specific case, with respect to bacterial load and environmental growth conditions, the extended challenge method might provide a tool for the evaluation of the duration of antimicrobial therapy required for an adequate therapy result.

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

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

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