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1 Title

2 Time course of biofilm formation by *Staphylococcus aureus* and *Staphylococcus epidermidis*
3 mastitis isolates

4

5 Authors

6 Oliveira, M.¹; Nunes, S. F.^{1,2}; Carneiro, C.¹; Bexiga, R.^{1,3}; Bernardo, F.¹ and Vilela, C. L.¹

7 ¹CIISA/Faculdade de Medicina Veterinária, Avenida da Universidade Técnica, 1300-477

8 Lisboa, Portugal

9 ²Presently at CIDC, Cambridge University, Cambridge, United Kingdom

10 ³Presently at University of Glasgow, Veterinary Faculty, Glasgow, United Kingdom

11

12 Corresponding author: Manuela Oliveira

13 Phone + 351 213852800; Fax + 351 213652889; email: moliveira@fmv.utl.pt

14

15 Abstract

16 Biofilm formation is considered a selective advantage for staphylococci mastitis isolates,
17 facilitating bacterial persistence in the udder. It requires attachment to mammary epithelium,
18 proliferation and accumulation of cells in multilayers and enclosing in a polymeric matrix,
19 being regulated by several *loci*. As biofilm formation can proceed through different pathways
20 and time ranges, its detection may differ according to the time of observation.

21 This study aimed at evaluating the time course evolution of biofilm production in
22 *Staphylococcus aureus* (n=26) and *Staphylococcus epidermidis* (n=29) mastitis isolates by
23 Fluorescent *In Situ* Hybridisation. Biofilm-forming ability increased with incubation time for
24 both species: for *S. aureus*, 34.6%, 69.2% and 80.8% of the isolates were able to produce
25 biofilm at 24, 48 and 72 hours, respectively. For *S. epidermidis*, 44.8%, 62.1% and 75.9% of the
26 isolates were biofilm-positive at 24, 48 and 72 hours, respectively. No significant difference
27 was found between species at each time point (Friedman's test, $p>0.05$). For *S. aureus*, although
28 a significant difference was found between 24 and 48 hours (Wilcoxon matched paired test,

1 $p<0.05$), no significant difference was found between 24 and 48 hours ($p>0.05$). For *S.*
2 *epidermidis*, significant differences were found between each time point ($p<0.05$).

3 Bacterial biofilms may impair eradication of chronic mastitis, rendering antibiotherapy less
4 effective. Detection of biofilm-forming ability in mastitis isolates may provide useful
5 information for the establishment of a more adequate therapeutic regimen, in view of the
6 antimicrobial concentrations required for bacterial control. However, it is essential that biofilm
7 formation time course is taken into consideration.

8

9 Key words: Bio film, time course, staphylococci, bovine mastitis

10

11 Introduction

12

13 Mastitis remains the most costly disease in dairy production, due to decrease milk production,
14 increased health care costs and increased culling and death rates (Melchior *et al.*, 2006b). It is
15 the most frequent cause for antimicrobial therapy in cows, being hard to eradicate independently
16 of a good antimicrobial susceptibility *in vitro* shown by the implicated microorganisms
17 (Bradley, 2002; Vasudevan *et al.*, 2003; Melchior *et al.*, 2006a; Clutterbuck *et al.*, 2007).

18 There are several organisms that may be responsible for bovine mastitis (Bradley, 2002), but
19 *Staphylococcus aureus* and *Staphylococcus epidermidis* remain two of the most commonly
20 isolated. These bacteria are able to form biofilms, highly organized multicelular complexes that
21 represent an important virulence factor in staphylococci (Cucarella *et al.*, 2001; Vasudevan *et*
22 *al.*, 2003; Fox *et al.*, 2005; Melchior *et al.*, 2006b; Clutterbuck *et al.*, 2007).

23 Biofilm formation requires the bacterial attachment to solid surfaces promoted by surface
24 proteins, the development of bacterial multilayers and their enclosing in a large
25 exopolysaccharide matrix (Baselga *et al.*, 1993; Cucarella *et al.*, 2001; Fox *et al.*, 2005; Lasa
26 and Penadés, 2006; Latasa *et al.*, 2006; Melchior *et al.*, 2006b; Clutterbuck *et al.*, 2007; Rohde
27 *et al.*, 2007). These structures impair the action of phagocytic cells from the host immune
28 system and of antimicrobial compounds, and release planktonic cells from the outer layers,

1 allowing the persistence of bacterial infections (Baselga *et al.*, 1993; Cucarella *et al.*, 2001;
2 Vasudevan *et al.*, 2003; Fox *et al.*, 2005; Cerca *et al.*, 2006; Chambers *et al.*, 2006; Frank and
3 Patel, 2006; Melchior *et al.*, 2006a; Melchior *et al.*, 2006b; Clutterbuck *et al.*, 2007; Harraghy *et*
4 *al.*, 2006). Such characteristics allow mastitis staphylococci with biofilm-forming ability to
5 effectively adhere and colonise the mammary gland epithelium, and establish persistent
6 infections (Baselga *et al.* 1993; Arciola *et al.*, 2001; Vasudevan *et al.*, 2003; Fox *et al.*, 2005;
7 Melchior *et al.*, 2006b).

8 Biofilm formation is regulated by several *loci* (Arciola *et al.*, 2001; Cucarella *et al.*, 2001;
9 Cucarella *et al.*, 2002; Beeken *et al.*, 2003; Vasudevan *et al.*, 2003; Fox *et al.*, 2005; Lasa and
10 Penadés, 2006; Melchior *et al.*, 2006b). Since biofilm formation can proceed through different
11 pathways and time ranges, its detection may differ according to the time of observation. Time
12 required for biofilm formation varies between bacterial strains and these differences should be
13 taken into consideration for the establishment of adequate antibiotherapy measures. The present
14 work aimed at evaluating the time course evolution of biofilm production in *Staphylococcus*
15 *aureus* and *Staphylococcus epidermidis* mastitis isolates, which would provide useful
16 information for the establishment of a more adequate therapeutic regimen.

17

18 Material and Methods

19

20 Bacterial strains

21 Fifty-five mastitis isolates of *S. aureus* (n=26) and *S. epidermidis* (n=29) were used in this study
22 (Tables 1 and 2). They belong to a collection of subclinical mastitis isolates from dairy cows of
23 12 commercial dairy farms located in the Ribatejo-Oeste area of Portugal (Bexiga *et al.*, 2005),
24 and were chosen on the basis of their prevalence in each farm. After isolation from milk
25 samples, bacteria were identified through their biochemical profile (API System, BioMérieux)
26 and kept frozen at -80°C until further processing.

27

1 Biofilm production

2 Biofilm production was evaluated *in vitro* by direct observation in bacterial suspensions by
3 Fluorescent *In Situ* Hybridisation (FISH). The FISH protocol was performed as described
4 elsewhere, with few modifications (Oliveira *et al.*, 2006). After treatment of the ten well teflon
5 slides (Heinz Herenz, Hamburg, Germany) used as hybridisation supports, ten μ l of overnight
6 bacterial suspensions in TSB (Tryptic Soy Broth, Oxoid, CM0129B) were placed in each well
7 and incubated in a humid chamber for 24, 48, and 72 hours, at 37°C, to allow biofilm formation.
8 Afterwards, the FISH protocol was performed, including bacterial fixation with
9 paraformaldehyde, membrane permeabilization by ethanol and lysostaphin, hybridisation with
10 the 16S rRNA oligonucleotide probes, stringency washes and observation of the hybridized
11 cells by fluorescence microscopy (Oliveira *et al.*, 2006). Two oligonucleotide probes (MWG-
12 Biotech, Ebersberg, Germany) were used: Sta (5'-TCCTCCATATCTCTGCGC-3'; *E. coli* 697),
13 specific for *Staphylococcus* spp. and labelled with rhodamine in the 5'-end, and Sau (5'-
14 GAAGCAAGCTTCTCGTCCG-3'; *E. coli* 69), specific for *S. aureus* and labelled with
15 fluorescein in the 5'-end (Kempf *et al.*, 2000).

16

17 Statistical analysis

18 The time course of biofilm formation was evaluated (Wilcoxon matched paired test) and the
19 results for both species were compared (Friedman's test).

20

21 Results

22

23 Biofilm formation

24 According to the analysis of the phenotypic biofilm expression by FISH, nine (34.6%) *S. aureus*
25 isolates revealed the ability to produce biofilm at 24 hours, while 18 (69.2%) isolates were
26 biofilm-positive at 48 hours, and 21 (80.8%) isolates were biofilm-positive at 72 hours (Table
27 1).

1 Regarding *S. epidermidis* mastitis isolates, 13 (44.8%) revealed the ability to produce biofilm at
2 24 hours, while 18 (62.1%) isolates were biofilm-positive at 48 hours, and 22 (75.9%) isolates
3 were biofilm-positive at 72 hours (Table 2).

4

5 Statistical analysis

6 According to the Friedman's test, no significant difference was found between species in terms
7 of biofilm-forming ability at each time point (24 hours: $p=0.405$; 48 hours: $p=0.763$; 72 hours:
8 $p=0.655$). According to the Wilcoxon Matched Paired Test, among the *S. aureus* isolates there
9 was a significant difference between the number of biofilm-positive isolates at 24 and 48 hours
10 ($p=0.003$) and between the number of biofilm-positive isolates at 24 and 72 hours ($p=0.001$).
11 The difference between the number of biofilm-positive isolates at 48 and 72 hours was
12 considered nonsignificant ($p=0.083$). Regarding the *S. epidermidis* isolates, a significant
13 difference was observed between the number of biofilm-positive isolates at 24 and 48 hours
14 ($p=0.025$), at 48 and 72 hours ($p=0.046$) and at 24 and 72 hours ($p=0.003$).

15

16 Discussion

17

18 Biofilm formation by staphylococci constitutes an important virulence factor in mastitis,
19 allowing the persistence of bacterial infections by impairing host immune cells, enhancing
20 intramammary bacterial adherence, protecting against milking shear forces, releasing planktonic
21 cells from the outer layers, and protecting from antimicrobial substances (Baselga *et al.* 1993;
22 Cucarella *et al.*, 2001; Vasudevan *et al.*, 2003; Fox *et al.*, 2005; Lasa and Penadés, 2006;
23 Melchior *et al.*, 2006a; Melchior *et al.*, 2006b).

24 Biofilm formation is regulated by several *loci* and can proceed through several pathways, so its
25 detection may differ according to the time of observation. In this study, we aimed at evaluating
26 the time course evolution of biofilm production in *S. aureus* and *S. epidermidis* mastitis isolates,
27 which may influence the success of the therapeutic regimens applied. We used FISH for

1 evaluation of biofilm-forming ability of other isolates from the same collection, with results
2 similar to the Congo Red Agar and optical density measurement methods, that are widely
3 applied (Oliveira *et al.*, 2006). This method was applied to TSB bacterial suspensions. Although
4 some authors stated that milk enhances biofilm formation (Cucarella *et al.*, 2002), Oliveira *et al.*
5 (2006) found no difference between the biofilm production by planktonic cells *in vitro* (TSB) or
6 *ex vivo* (milk) growth conditions.

7 According to the phenotypic analysis of biofilm expression by FISH, one third of the *S. aureus*
8 mastitis isolates revealed biofilm-forming ability at 24 hours, and this percentage increased with
9 the incubation time (Table 1). Regarding the *S. epidermidis* isolates, the number of positive
10 isolates was maximal at 24 hours, accounting for almost half of the biofilm-positive strains.
11 Similarly to the *S. aureus* results, this percentage increased with the incubation time (Table 2).
12 Other authors have already evaluated the biofilm-forming ability of bovine mastitis
13 staphylococci (Baselga *et al.*, 1993; Arrizubieta *et al.*, 2004; Fox *et al.*, 2005; Tormo *et al.*,
14 2005), but it should be noticed that in the majority of the studies the capacity of forming biofilm
15 was evaluated after a 24 hours incubation, which may be insufficient for some isolates (Arciola
16 *et al.*, 2001).

17 The statistical analysis of the results showed that slime production appears equally common for
18 the *S. aureus* and *S. epidermidis* under study, since there were no significant difference between
19 species regarding biofilm-forming ability at each time point, which is in accordance with
20 Arciola *et al.* (2001), Beeken *et al.* (2003) and Oliveira *et al.* (2006). A significant increase in
21 the number of biofilm-positive *S. aureus* isolates was observed between 24 and 48 hours, but no
22 significant differences were found between 48 and 72 hours. Regarding *S. epidermidis*, the
23 increase in the number of isolates with biofilm-forming ability was significant between 24 and
24 48 hours, and also between 48 and 72 hours. These results are not in agreement with the
25 findings of other authors, which stated that biofilm production is slower in *S. aureus* (48-72
26 hours) than in *S. epidermidis* (24 hours) (Arciola *et al.*, 2001; Cucarella *et al.*, 2001; Vasudevan
27 *et al.*, 2003).

1 Because intramammary infections can become chronic, with an inherent resistance to
2 antimicrobial therapy, knowledge concerning the time course of virulence factors expression is
3 essential for the adequate application of therapeutic measures.

4 5 Conclusion

6
7 This study revealed that the majority of *S. aureus* biofilm-producers can be detected in 48 hours
8 cultures, and that a 72 hours incubation period is more adequate for *S. epidermidis*. The
9 knowledge about time course of biofilm expression may contribute to improve the results of
10 antimicrobial mastitis therapy.

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13
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18 23226/05).

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4

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Table 1 – Time course of biofilm formation by *Staphylococcus aureus* mastitis isolates.

Isolate*	Biofilm-forming ability		
	24 h incubation	48 h incubation	72 h incubation
A395AE	Positive	Positive	Positive
C986	Positive	Positive	Positive
C842AD	Positive	Positive	Positive
C978AD	Negative	Positive	Positive
C23PD	Positive	Positive	Positive
C1082PE	Negative	Negative	Negative
C864PE	Positive	Positive	Positive
D674	Negative	Positive	Positive
D8927PD	Negative	Positive	Positive
D9236AD	Positive	Positive	Positive
D176AD	Negative	Positive	Positive
D43AE	Negative	Positive	Positive
D8330AD	Negative	Negative	Positive
D8927PE	Positive	Positive	Positive
D2811PD	Negative	Positive	Positive
D9236AE	Positive	Positive	Positive
D129PE	Negative	Negative	Positive
D7418AD	Negative	Positive	Positive
F9268AD	Positive	Positive	Positive
I65AE	Negative	Negative	Negative
I570PD	Negative	Negative	Positive
I516PD	Negative	Negative	Negative
I100AD	Negative	Positive	Positive

I709AD	Negative	Negative	Negative
Z6053	Negative	Negative	Negative
L83PE	Negative	Positive	Positive
n=	9 (34.6%)	18 (69.2%)	21 (80.8%)

* Belonging to a collection of subclinical mastitis field isolates from our laboratory.

Table 2 – Time course of biofilm formation by *Staphylococcus epidermidis* mastitis isolates.

Isolate*	Biofilm-forming ability		
	24 h incubation	48 h incubation	72 h incubation
A398	Negative	Positive	Positive
B9003	Negative	Positive	Positive
B6055AD	Negative	Negative	Positive
B7016PD	Negative	Positive	Positive
B7218PE	Negative	Positive	Positive
B8210PD	Positive	Positive	Positive
F7125	Positive	Positive	Positive
F7029PD	Positive	Positive	Positive
F7029PE	Negative	Negative	Positive
F7034PE	Positive	Positive	Positive
F7036AE	Positive	Positive	Positive
F7059AD	Negative	Negative	Negative
F7347PD	Positive	Positive	Positive
F7481PD	Negative	Positive	Positive
F7519PE	Negative	Negative	Positive
F7706AE	Positive	Positive	Positive
F7800PD	Positive	Positive	Positive
F7060PD	Negative	Negative	Negative

F7970PE	Positive	Positive	Positive
F8149AE	Positive	Positive	Positive
F9326AD	Positive	Positive	Positive
F9336AD	Negative	Negative	Negative
H231	Negative	Negative	Negative
H159PE	Negative	Negative	Negative
H162PD	Negative	Negative	Negative
J8134AE	Positive	Positive	Positive
J8097PD	Negative	Negative	Negative
K2939AD	Negative	Negative	Positive
K3053PE	Positive	Positive	Positive
n=	13 (44.8%)	18 (62.1%)	22 (75.9%)

* Belonging to a collection of subclinical mastitis field isolates from our laboratory.