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In vitro ability of mastitis causing pathogens to form biofilms

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This Research Communication describes the study of *in vitro* biofilm formation of mastitis causing pathogens. Biofilms are communities of bacteria that are attached to a surface and to each other and are embedded in a self-produced matrix of extracellular polymeric substances. Biofilm formation is an important virulence factor that may result in recurrent or persistent udder infections and treatment failure through increased resistance to antibiotics and protection against host defences. In the present study 252 bacterial isolates from milk samples from bovine udder quarters with intramammary infections were examined with Congo Red agar (CRA) method and tube method (TM) for their ability to form biofilms. Both tests revealed a high number of biofilm-positive strains. Literature reports that the cure rates for *Staphylococcus aureus* infected udders are lower (27%) in comparison to cure rates of Streptococcus uberis (64–81%) or coagulase-negative staphylococci (CNS) mastitis (80–90%). The findings of the present study suggest that biofilm formation is not the main factor for the differences in cure rates of the various bacteria genera, because all tested pathogen groups showed a similarly high proportion of biofilm formation. Further research is needed to detect microbial biofilms on bovine udder epithelia.

Keywords: Biofilm, *Staphylococcus aureus*, Mastitis, Intramammary infection.

Mastitis in dairy cow herds is a serious problem for milk producers because it leads to decreased milk production, high costs for medical treatment, and an increased culling and death rate (Oliveira et al. 2006). In the majority of cases, intramammary infections are treated with antibiotic agents. Biofilms protect microorganisms against these antibiotic agents (Bose et al. 2009), phagocytosis (Høiby et al. 2011), and sanitisers (Donlan & Costerton, 2002; Høiby et al. 2011). The ability of bacteria to produce biofilms leads to difficulties with pathogen elimination, which can give rise to persistent infections (Oliveira et al. 2006; Høiby et al. 2011; Darwish & Asfour, 2013). Resistance to antimicrobial agents results, amongst other factors, from the retardation of antibiotic diffusion through the biofilm matrix, an increased rate of mutation, the production of enzymes that degrade antibiotics, the presence of dormant bacterial cells with low metabolic activity and increased doubling times in the inner layers of biofilms (Høiby et al. 2010).

The development of a biofilm can be influenced by oxygen and iron limitation, high osmolarity (Otto, 2008) and subinhibitory concentrations of some antibiotics (Høiby et al. 2010). Biofilm formation takes place in two steps: In the first step, planktonic bacteria attach reversibly to a surface. Afterwards, the binding becomes irreversible and the bacteria proliferate and produce a polymer matrix (Høiby et al. 2011). In mature biofilms the detachment of contagious emboli occurs. This emboli can spread in a patient body and can be responsible for disseminated infections (Donlan & Costerton, 2002).

The aim of the present study was to reveal to what extent different mammary pathogenic bacterial strains are able to form biofilms.

Material and methods

Bacterial stains

For the investigation, 32 *Staphylococcus* (*S.*) *aureus,* 36 coagulase-negative staphylococci (CNS), 29 *Streptococcus* (*Str.*) *dysgalactiae, 37 Str. Uberis, 28* coryneform bacteria, 31 *Escherichia* (*E.*) *coli* 32 *Klebsiella* spp. and 28 coliform bacteria (other than *E. coli* and *Klebsiella* spp.) isolates, recovered from quarter milk samples of cows with subclinical and clinical intramammary infections from 66 German farms, were used.

In short, for the isolation 0·01 ml of each well mixed sample was streaked onto a quadrant of an esculin blood

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agar plate (Oxoid, Am Lippeglacis 4, 46483 Wesel, Germany). The plates were incubated aerobically at 37 °C for 48 h. Bacteria were identified by their cultural characteristics on esculin blood agar, Gram staining, catalase reaction, and biochemical tests, as previously described by Mansion-de Vries et al. (2014).

Detection of biofilm formation

Biofilm formation ability was determined by two different methods: Congo Red agar (CRA) method and tube method (Bose et al. 2009).

Congo Red agar method

The medium consists of brain-heart broth (37 g/l) (Merck, Frankfurter Straße 250, 64293 Darmstadt, Germany), glucose (5 g/l) (Roth, Schoemperlenstraße 1, 76185 Karlsruhe, Germany), agar agar (10 g/l) (Merck, Frankfurter Straße 250, 64293 Darmstadt, Germany), and Congo Red dye (0·8 g/l) (Applichem, Ottoweg 4, 64291 Darmstadt, Germany). Congo Red was dissolved in aqua dest. and autoclaved at 121 °C for 15 min. Then it was added to autoclaved brain-heart infusion agar with glucose at 55 °C.

The Congo Red agar plate was inoculated with a sterile inoculation loop with a test organism cultivated on esculin blood agar plate, afterwards it was aerobically incubated at 37 °C for 48 h. Black colonies with a dry consistency and rough surface and edges indicate slime production. Both black colonies with smooth, round, and shiny surfaces and red colonies of dry consistency with rough edges and surfaces were categorised, according to Darwish & Asfour (2013), as intermediate slime producers. Red colonies with smooth, round, and shiny surfaces were considered negative for slime production (Darwish & Asfour, 2013).

Tube method

The tube test was performed according to Bose et al. (2009) with slight modifications.

1 ml autoclaved Tryptic soy broth (Merck, Frankfurter Straße 250, 64293 Darmstadt, Germany) was filled into sterile plastic cuvettes (Omnilab, Robert-Hooke-Straße 8, 28359 Bremen, Germany) in duplicate and one of them was inoculated with an inoculation loop full of bacteria cultivated on esculin blood agar plates. The second cuvette was used as negative control. Suspensions were incubated at 37 °C for 24 h and after that the liquid was decanted and the cuvettes were washed once with water and stained with a safranine solution (1·8 g/l) (Merck, Frankfurter Straße 250, 64293 Darmstadt, Germany). Then the stain was washed away with water. Biofilm-positive strains give a visible rose to red film on the inner wall of the cuvettes.

Statistical analysis

SPSS 23·0 (IBM Corp.) software was used to compare sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) on CRA method based on the tube method (TM) as reference.

Results and discussion

Slime production on Congo Red agar (CRA)

The results of biofilm formation by the different bacterial species are given in Table 1.

In total 252 isolates were included into the study. 25·8% (65) of the strains produced black colonies with dry consistency and rough surfaces. Together with the 140 strains with an intermediate result, 81·3% of the examined strains were positive for slime production. Only 18·7% (47) were classified as non-producers. *Str. dysgalactiae* was almost completely positive for slime production on CRA. It was found that slime production appears equally common in *E. coli*, coryneform bacteria, and *Str. uberis*.

Biofilm formation by the tube method (TM)

As shown in Table 2, 71·8% of the isolates were revealed to be biofilm producers. However, the production level varied in the examined species. Both *E. coli* and coliform bacteria other than *E. coli* and *Klebsiella* spp. are able to generate biofilms in around 90% of cases, whereas only 42·9% of the coryneform bacteria are positive for biofilm formation according to TM. Likewise, less than half (45·9%) of *Str. uberis* isolates forms a biofilm on the inner wall of the cuvette.

Comparison of the CRA and tube methods

In total, CRA has a sensitivity of 73% and a specificity of 25%, when TM is considered as reference method. The PPV and NPV were calculated to be 75·1 and 23%, respectively.

Biofilm formation is an important virulence factor that may cause recurrent or persistent mastitis by impairing the host immune defence and through the protection of antimicrobial substances (Oliveira et al. 2006; Darwish & Asfour, 2013). The aim of the present study was to evaluate the biofilm-forming ability of 252 bacterial strains from 8 different udder pathogen groups on CRA and on the walls of cuvettes. It was shown that 81·3% (CRA) and 71·8% (TM) of the analysed isolates have the ability to produce slime/biofilms. From 32 *S. aureus* strains, 77·4% were positive on CRA. A similar result has been reported in the study of Darwish & Asfour (2013), who found 67·5% positive *S. aureus* strains on CRA. Lower positivity rates have been reported by Oliveira et al. (2006), Bose et al. (2009) and Fabres-Klein et al. (2015). One explanation for the higher rate of positive results found in our study and also in the work of Darwish & Asfour (2013) could be the distinct categorisation of positive, intermediate, and negative results. All intermediate results were assessed as slime producers in these studies, whereas Bose et al. (2009) and Fabres-

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Table 1. Biofilm formation of 252 udder pathogenic bacteria on Congo Red agar (CRA)

	% (no.) of isolates			
Species/Pathogen group (no.)	Positive results Dry black colonies	Intermediate results Smooth black and dry red colonies	Negative results Smooth red colonies	Total positive (positive + intermediate results)
S. aureus (31)	3·2 (1)	74·2 (23)	22·6 (7)	77·4 (24)
CNS (36)	5·6 (2)	58·3 (21)	36·1 (13)	63·9 (23)
Str. dysgalactiae (29)	96·6 (28)	0 (0)	3·4 (1)	96·6 (28)
Str. uberis (37)	86·5 (32)	13·5 (5)	0 (0)	100 (37)
Coryneforms (28)	7·1 (2)	92·9 (26)	0 (0)	100 (28)
E. coli (31)	0 (0)	100 (31)	0 (0)	100 (31)
Klebsiella spp. (32)	0 (0)	53·1 (17)	46·9 (15)	53·1 (17)
Other coliforms (28)	0 (0)	60·7 (17)	39·3 (11)	60·7 (17)
Total (252)	25·8 (65)	55,5 (140)	18·7 (47)	81·3 (205)

Table 2. Biofilm formation of 8 udder pathogen groups according to the tube method

	% (no.) of isolates	
Species/Pathogen group (no.)	Positive	Negative
S. aureus (31)	74·2 (23)	25·8 (8)
CNS (36)	80·6 (29)	19·4 (7)
Str. dysgalactiae (29)	69·0 (20)	31·0 (9)
Str. uberis (37)	45·9 (17)	54·1 (20)
Coryneforms (28)	42·9 (12)	57·1 (16)
E. coli (31)	90·3 (28)	9·7 (3)
Klebsiella spp. (32)	84·4 (27)	15·6 (5)
Other coliforms (28)	89·3 (25)	10·7 (3)
Total (252)	71·8 (181)	28·2 (71)

Klein et al. (2015) considered only black colonies with a dry and crystalline consistency as positive results. Oliveira et al. (2006) used only the colony colour for the evaluation.

Fabres-Klein et al. (2015) ascribed the lower positivity rate to CRA, which can only detect slime, the extracellular polymeric substance (EPS). The black colour is probably formed as a result of association between a thick exopolysaccharide layer and the Congo Red dye. Bacterial cells that are surrounded by thinner EPS layers build lighter colours, resulting in the inability of CRA to differentiate bacteria with a thin polysaccharide layer and slime-negative strains (Fabres-Klein et al. 2015).

With the tube method, biofilms, which are defined as a population of bacterial cells that are attached to a surface and surrounded by a matrix (Donlan & Costerton, 2002) can be verified. It has been reported that TM shows the best sensitivity and specificity (both 100%) in comparison to PCR (Oliveira & de Cunha, 2010). Therefore, TM was used in the present work as reference for CRA.

Our results for biofilm-positive *S. aureus* strains (74·2%) are not in agreement with the result of Bose et al. (2009), who detected 42·5% biofilm-forming ability across *S. aureus* strains. The results obtained by Oliveira & de Cunha (2010) for CNS with CRA method (73%) and tube method (82%) agree with our findings of 63·9% for CRA method and 80·6% for tube method. Darwish & Asfour (2013) had similar outcomes for CNS on CRA (72·1%).

In this study, a sensitivity of 73·0% and a specificity of 25·0% for the CRA method, with TM as the reference, were detected. The CRA method is easy and fast to perform, but it is imprecise in the identification of biofilm-negative isolates when compared to results obtained with TM. As a consequence CRA method cannot be recommended to be used alone for the detection of biofilm production. The target of CRA and TM is the phenotypic expression of biofilm formation; with PCR genotypically positive strains could be identified which are phenotypically negative for biofilm production (Oliveira et al. 2006). However, PCR-positive strains should be considered to have the potential for biofilm production through the expression of *ica* genes, but it is recommended that biofilm formation should be confirmed by one additional phenotypic method (Oliveira & de Cunha, 2010).

The data obtained in the present study show that a high number of mastitis pathogens possess the ability to produce biofilms. This important virulence factor can lead to persistence of bacteria in mammary glands and can lead to chronic mastitis because of the low efficiency of antibiotic treatment. However, the inferior cure rates of *S. aureus* (27%) (Steele & McDougall, 2013) cannot be explained by the fact of biofilm formation alone. A majority of the tested pathogens showed biofilm formation. Nevertheless, the cure rates of *Str. uberis* (64–81%), *E. coli* (85–93%) (Swinkels et al. 2014) and CNS (80–90%) (Pyörälä & Taponen, 2009) are distinctly higher than the cure rates of *S. aureus*. The ability of *S. aureus* to form biofilms is thus not the main cause for the low cure rates.

However, no proof for *in vivo* biofilms in mammary tissue has been brought so far. In further studies a focus should be laid on the verification of *in vivo* biofilm formation in udder quarters with intramammary infections.

Conclusion

The present study revealed that a high percentage of mammary pathogenic bacterial strains are able to build biofilms. Due to these results, it can be concluded that biofilm formation is not the main cause for the low cure rates in *S.*

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aureus mastitis. Biofilms are difficult to eradicate, so in future the focus has to be placed on effective prophylaxis and a suitable treatment regime.

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