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Therapeutic effects of a combined antibiotic-enzyme treatment on subclinical mastitis in lactating dairy cows

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ABSTRACT: The objective of this study was to evaluate a combined antibiotic-enzyme therapy for *Staphylococcus aureus* mastitis and biofilm formation. A total of 141 cases of *S. aureus* chronic mastitis from three farms were divided into two groups: the control group (n = 54) were treated with Nafpenzal® ointment; the enzyme + antibiotic group (n = 87) were treated with Nafpenzal® plus an enzymatic ointment (MastiVeyxym®). Quantitative determination of biofilm formation was determined using a colorimetric microplate assay and the detection of *ica* genes by PCR. Enzyme + antibiotic therapy did not significantly improve cure rates compared to control (48.3 vs 38.9%). The cure rate for infections caused by biofilm-positive strains was 42.6 and 46.6% for control and enzyme + antibiotic groups, respectively ($P > 0.05$). Comparison of cure rates between farms showed a relationship with somatic cell count (SCC), parity and oxacillin resistance. 79.4% of the isolates produced biofilm and antibacterial resistance rates for oxacillin, penicillin and streptomycin were 25.5, 71.6 and 95.7%, respectively. These results indicate that while the increase in mastitis cure rate using enzymatic therapy was not significant, this treatment could be useful in some situations.

Keywords: mastitis; *Staphylococcus aureus*; biofilm; enzyme-antibiotic treatment; genomic DNA; *ica* genes

Staphylococcus aureus is the most common cause of contagious bovine mastitis which generally manifests as a chronic recurrent infection exhibiting resistance to therapy (Zecconi et al. 2006). One of the most convincing hypotheses to explain the difficulties in treating recurrent infections might be related to the ability of staphylococci to form biofilms in infected tissues (Melchior et al. 2006). Biofilms are complex communities of microorganisms attached to surfaces and surrounded by a matrix of extracellular polymer (Davey and O'Toole 2000). Biofilm formation is an important element determining the pathogenicity and antimicrobial resistance of bacteria (Snel et al. 2014; Castelani et al. 2015; Krewer et al. 2015). Biofilms can decrease or block the penetration of antibiotics by formation of a physical barrier or can bind to antibiotics with their negatively charged exopolysaccharides. Furthermore, the altered physiology of bacterial cells in biofilms and an improper response of the immune system can lead to difficulties in biofilm eradication (Melchior et al. 2006). Bacteria in biofilms may be 10 to 1000 times more resistant to antimicrobial agents as compared to the planktonic form (Melchior et al. 2006). Biofilm formation consists of two stages; the first stage comprises attachment of cells to a surface by adhesion factors and creation of a primary cell aggregation. The second stage is characterised by cell multiplication and formation of a mature multilayer structure (Melchior et al. 2006). It was found that the intracellular adhesion (*ica*) operon is essential for control of biofilm production (Grinholc et al. 2007). We previously described a significantly altered prevalence rate of microbial surface components that recognise adhesive matrix molecules (MSCRAMM) and of *ica* genes between isolates with no biofilms and biofilm-producer isolates (Khoramian et al. 2015). The *ica* locus consisting of the *ica*ADBC genes encodes the proteins mediating the synthesis of polysaccha-

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ride intercellular adhesions (PIA) (Arciola et al. 2001; Vasudevan et al. 2003). The biofilm matrix is a collection of polysaccharides, proteins, nucleic acids, glycoproteins and phospholipids (Czaczyk and Myszka 2007). With this in mind, it is clear that enzymes can be used for degradation of biofilm (Johansen et al. 1997; Berg et al. 2001; Mecikoglu et al. 2006; Lu and Collins 2007; Chaignon et al. 2007). Enzymatic activity can be directed to the biofilm matrix, allowing better penetration of the subsequent antimicrobial agents used or can be targeted towards pathogen cell wall components in order to cause their lysis (Johansen et al. 1997; Kaplan et al. 2004).

The aim of this study was to evaluate the role of biofilm formation on the refractoriness of treatment of *S. aureus* subclinical mastitis cases and to test whether a combined antibiotic-enzymatic therapy could improve cure rates of *S. aureus* subclinical mastitis during lactation.

MATERIAL AND METHODS

Animals. The experiment was performed on three large dairy farms in Tehran, Iran with more than 100 CFU/ml of *S. aureus* in bulk milk tank. The cows were milked three times a day and subjected to pre- and post-milking teat disinfection. Cows were housed in loose barns and fed with total mixed ration (TMR). Individual milk samples were collected from cows with a cow composite milk SCC above 400 000 cells/ml for two consecutive months. Cows with subclinical *S. aureus* mastitis in only one quarter and with lactation numbers one to seven (median = three) and lactation months two to ten (median = five) were enrolled in the study. They had no concurrent disease and received no clinical mastitis or anti-infectious and anti-inflammatory treatments within the preceding 14 days.

Sampling and Bacterial isolation. A total of 141 isolates of *S. aureus* were collected from milk samples of cows with subclinical mastitis. Sampling, culture and identification of the bacteria were performed according to the National Mastitis Council (NMC). *S. aureus* was identified by Gram staining, production of coagulase, catalase, DNase, oxidation and fermentation of mannitol. To confirm the identity of *S. aureus*, the *nuc*A gene was amplified by a PCR-based method, using the primers listed in Table 1. A quarter milk sample was considered to be contaminated when three or more bacteria could be isolated. Another sampling was performed from the affected quarters 14 and 28 days after the last treatment for bacteriological investigations. A bacteriological cure was signified if the samples taken from the affected quarter were negative.

Treatment groups. Cows were randomly allocated into two treatment groups: (1) The control group (n = 54) were treated only with intramammary infusion of 100 mg sodium nafcillin with 180 mg sodium penicillin, and 100 mg dihydro streptomycin sulphate (Nafpenzal MC; Intervet International, Boxmeer, Netherlands) every 24 h for three consecutive days. (2) The enzyme + antibiotic group (n = 87) were treated with Nafpenzal Ointment plus a commercial preparation consisting of 8 mg chymotrypsin, 8 mg trypsin, 4 mg papain, 100 000 IU vitamin A and 120 mg vitamin E (MastiVeyxym; Veyx-Pharma GmbH, Germany) every 24 h for three consecutive days.

Quantification of biofilm production. Quantitative determination of biofilm-forming capacity was determined using a colorimetric microtiter plate Assay as described by Peeters et al. (2008) with slight modifications. Briefly, *S. aureus* isolates were grown overnight at 37 °C in trypticase soy broth (TSB) (Merck, Darmstadt, Germany) and then the bacterial suspensions were diluted

Table 1. The oligonucleotide primers used in this study

Gene	Primer sequence (5' to 3')	References
nucA-F	5'-CTGGCATATGTATGGCAATTGTT-3'	Sahebekhtiari et al. 2011
nucA-R	5'-TATTGACCTGAATCAGCGTTGTCT-3'	
icaA-F	5'-ACACTTGCTGGCGCAGTCAA-3'	Arciola et al. 2001
icaA-R	5'-TCTGGAACCAACATCCAACA-3'	
icaD-F	5'-ATGGTCAAAGCCCAGACAGAG-3'	
icaD-R	5'-AGTATTTTCAATGTTTAAAGCAA-3'	

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1 : 100 in medium. One hundred and fifty µl of this cell suspension were used to inoculate sterile flat-bottomed 96-well polystyrene microtiter plates. Subsequent to an incubation period of 24 h at 37 °C without shaking, wells were gently washed three times with 200 µl of phosphate buffered saline (PBS) and dried in an inverted position. For fixation of biofilms, 150 µl of 99% methanol were added and after 15 min supernatants were removed and the plate was air-dried. Then, 150 µl of crystal violet 1% (CV) were added to all wells. After 20 min the excess CV was removed by washing the plate under running tap water. Finally, bound CV was released by adding 150 µl of 33% acetic acid. The optical density (OD) of each well was measured at 590 nm using a microtiter plate reader. All the assays were repeated three times. As a control, uninoculated medium was used to determine background OD. The cut-off OD (ODc) was defined as three standard deviations above the mean OD of the negative control. According to the results of the microtiter plate test, the isolates were classified into the following four categories based on the optical density: non-biofilm producers (OD test < ODc), weak biofilm producers (ODc < OD < 2 × ODc), moderate biofilm producers (2 × ODc < OD < 4 × ODc), and strong biofilm producers (4 × ODc < OD).

Detection of *ica* genes. Whole genomic DNA from cultured strains was prepared using the phenol-chloroform method. *icaA* and *ica*D genes were detected by a PCR method using the specific oligonucleotide primers listed in Table 1. Amplification was performed in a final volume of 25 µl containing 300nM of each primer, 1 × reaction buffer, 2.5mM $MgCl_2$, 0.2mM dNTPs, 5 µl of template DNA and 1.5 IU of Taq polymerase (Fermentas, UAB, Lithuania). The PCR conditions consisted of a pre-denaturation step at 94 °C for 5 min, followed by 30 cycles of 45 s at 94 °C, 45 s at 54 °C and 40 s at 72 °C. A final extension step was performed at 72 °C for 5 min. Amplified products were analysed by electrophoresis on 1% agarose gels. DNA bands were visualised by staining with ethidium bromide and were photographed under UV illumination.

Antimicrobial susceptibility testing. *In vitro* susceptibility of *S. aureus* isolates was determined using the disk agar diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). The following antibiotics were tested: penicillin (10 units), oxacillin (1 µg) and streptomycin (10 µg). *S. aureus* ATCC 29213 was used as the reference strain for quality control purposes.

Statistical analysis. SAS software (version 9.2; SAS Institute, Cary, NC) was used for statistical analysis. Differences in cure rates and the prevalence of genes between *S. aureus* strains and farms were calculated using the chi-square test. Thereafter, data were analysed with a multivariate logistic regression using the LOGISTIC procedure of SAS. Parity, days in milk, SCC, treatment, presence of *ica* genes, biofilm formation and antimicrobial resistance were considered as covariates in the models to examine effects on cure rates. A *P*-value of ≤ 0.05 was considered statistically significant.

RESULTS

There was no significant difference in cure rates between the two groups (48.3 vs 38.9%) (Table 2),

Table 2. Comparison of cure rate, biofilm formation, *ica* gene prevalence and antimicrobial resistance between the two treatment protocols

Group	Cure rate	icaA+	icaD+	Biofilm+	Mean score value of biofilm	Antibiotic resistance		
						oxacillin	penicillin	streptomycin
Enzyme + antibiotic	48.3% (42/87)[a]	66.7% (58/87)[a]	64.4% (56/87)[a]	74.7% (65/87)	0.98	24.1% (21/87)	70.1% (61/87)	96.6% (84/87)
Control	38.9% (21/54)[a]	64.8% (35/54)[a]	61.1% (33/54)[a]	87% (47/54)	1.20	27.7% (15/54)	74% (40/54)	94.4% (51/54)
Total	44.7% (63/141)	66% (93/141)	63.1% (89/141)	79.4% (112/141)	1.13	25.5% (36/141)	71.6% (101/141)	95.7% (135/141)

[a]same letters in each columns are not significantly different from each other ($P > 0.05$)

icaA+/icaD+= presence of *ica*A/*ica*D gene in *S. aureus* strains. Biofilm+: all weak, moderate or strong biofilm producer. Mean score value of biofilm: score 0 = non biofilm producers, score1 = weak biofilm, score 2 = moderate biofilm, score 3 = strong biofilm

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Table 3. Comparison of cure rates, ica gene prevalence, capacity of biofilm formation and antibiotic resistance between farms

Farm	Cure rate		Parity	DIM	SCC×1000	Biofilm positive	Mean score value of biofilm	icaA	icaD	Antibiotic resistance		
	enzyme + antibiotic	control								nafcillin	penicillin	streptomycin
Farm A (n = 40)	50% (12/24)[a]	62.5% (10/16)[a]	2.7	229	1083	100% (40/40)[a]	1.45	97.5% (39/40)[a]	95% (38/40)[a]	2.5% (1/40)[a]	100% (40/40)[a]	100% (40/40)[a]
Farm B (n = 38)	43.5% (10/23)[a]	20% (3/15)[b]	4.2	173	1388	55.3% (21/38)[b]	0.89	10.5% (4/38)[b]	10.5% (4/38)[b]	7.9% (3/38)[a]	26.3% (10/38)[b]	86.8% (33/38)[b]
Farm C (n = 63)	50% (20/40)	34.8% (8/23)[ab]	2.8	192	1077	81% (51/63)[b]	1.08	79.4% (50/63)[c]	74.6% (47/63)[c]	50.8% (32/63)[b]	81% (51/63)[c]	96.8% (61/63)[ab]

[a,b,c]means in the same column with different superscripts are significantly different ($P < 0.05$). DIM: days in milk. Biofilm positive: all weak, moderate or strong biofilm producer. Mean score value of biofilm: score 0 = non biofilm producer, score 1 = weak biofilm, score 2 = moderate biofilm, score 3 = strong biofilm; icaA/icaD = presence of *ica*A/*ica*D gene in *S. aureus* strains

or among the three farms, irrespective of the treatment protocol ($P > 0.05$). Comparison of the two treatment strategies on each farm did not show any significant difference ($P > 0.05$). Further, there were no significant differences between the icaA and icaD prevalences, biofilm formation capacity and antimicrobial resistance patterns in isolates from both treatment groups (Table 2).

In the current study, cure rates in biofilm-positive strains were 42.6 and 46.6% for control and Enzyme + antibiotic groups. respectively ($P > 0.05$). On farm B, with the lowest cure rate (34.2%), the lowest prevalence of *ica* genes, biofilm formation capacity, penicillin and streptomycin resistance rates in *S. aureus* isolates, and days in milk (DIM) were noted; however, these isolates exhibited the highest SCC, parity, and oxacillin resistance. Furthermore, farm A with the highest cure rate (55%) had the highest prevalence of *ica* genes, biofilm formation and penicillin and streptomycin resistance and DIM but had the lowest SCC, parity and oxacillin resistance (Table 3). The evaluation of the effect of independent variables on cure rates by logistic regression did not reveal any significant differences ($P > 0.05$). Approximately 79.4% (112/141) of the isolates produced biofilm. In the biofilm-producing strains, 61.6% (69/112) of isolates were weak, 33.9% (38/112), while 4.5% (5/112) were moderate and strong, respectively. Antibacterial resistance rates for oxacillin, penicillin and streptomycin were 25.5%, 71.6% and 95.7% by the disk agar diffusion method, respectively.

DISCUSSION

Mastitis caused by *S. aureus* is one of the most common types of chronic mastitis. The poor predictive capacity of *in vitro* antibiotic susceptibility tests for bacteriological treatment, the higher cure rate of acute infections than chronic infections and the usefulness of extended therapy for improving cure rates are often attributed to bacterial biofilm formation. Many studies have found alternatives strategies for eradication of biofilms or for prevention of biofilm formation. Enzymes represent an alternative option for the degradation of bacterial biofilms (Johansen et al. 1997; Berg et al. 2001). For many biofilms, proteins are the largest percentage of the total material (Molobela et al. 2010), so proteases are able to cleave these proteins at specific amide bonds, effectively destroying the physical structure of a biofilm (Leslie 2011).

The prevalence of *ica*A and *ica*D genes in our study was 66 and 63.1%, respectively. The evaluation of the effect of independent variables on cure rates by logistic regression did not reveal any significant difference ($P > 0.05$). Also, comparison of the cure rates among the three farms revealed no effect of the high prevalence of *ica* genes or biofilm formation capacity on cure rate.

The results of this study contradict those of previous studies showing that formation of biofilm has an impact on the efficacy of antimicrobial therapy and the subsequent outcome of infection (Gotz 2002; Parsek and Fuqua 2004; Castelani et al. 2015;

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Krewer et al. 2015). This may be due to the fact that biofilm production in the mammary tissue is different from the *in vitro* situation. Also, it was shown that other factors like SCC, parity and oxacillin resistance are more important than the presence of *ica* genes and biofilm formation capacity.

The results of the present study show that the concomitant *in vivo* use of a mixture of enzymes with antibiotics slightly improved cure rate; however, this improvement was statistically non-significant. Also, there was no statistically significant difference between cure rates of biofilm-positive strains in the two groups. Some *in vitro* studies have suggested the possibility of using enzyme in removing biofilms (Johansen et al. 1997; Berg et al. 2001; Kaplan et al. 2004). Chaignon et al. (2007) showed that the enzymatic detachment of staphylococcal biofilms depends on the nature of their constituents and varies between clinical isolates; they tested the effect of different enzymes on biofilms and suggested a complex mixture of dispersin B and a protease (like trypsin) to remove the biofilms of a variety of staphylococcal strains. Other *in vitro* studies showed that proteolytic enzymes (Selan et al. 1993) or lysostaphin (Walencka et al. 2005; Walencka et al. 2006) could significantly enhance the activities of antibiotics against biofilms.

There are a few *in vivo* studies on the use of enzymes for eradicating infection caused by biofilms. Kokai-Kun et al. (2009) showed that lysostaphin administered at 15 mg/kg in combination with 50 mg/kg nafcillin, three times per day for four days in mice, eradicated *S. aureus* biofilms from implanted catheters and eliminated heart and liver infections in *S. aureus*-infected mice (Kokai-Kun et al. 2009). Mecikoglu et al. (2006) showed that serratiopeptidase was effective for eradicating infection caused by biofilm-forming bacteria, and also that the anti-biofilm property of the enzyme may enhance antibiotic efficacy in the treatment of staphylococcal infections (Mecikoglu et al. 2006). The different results found in the present study could be due to the type of enzyme. Also, it was indicated that other factors like SCC, parity and antimicrobial resistance are more important than biofilm formation capacity. This probably suggests that enzymes increase the cure rate by breaking down pus, clot and fibrin and by clearing the milk duct and without any effect on biofilm. Some investigators showed that the use of enzyme is not useful in the planktonic condition of growth (Selan et al. 1993; Johansen et al. 1997; Berg et al. 2001; Kaplan et al. 2004). We evaluated biofilm formation capacity outside of the mammary gland; obviously, our *in vitro* conditions may differ from the *in vivo* situation.

In conclusion, the data described in the present paper indicate that biofilm-forming capacity is not the most important predictive factor associated with cure rate after treatment of subclinical mastitis caused by *S. aureus*. Moreover, the use of enzymatic ointment together with antibiotics slightly, but non-significantly, improved the cure rate. Further research is needed to test the effects of other enzymes on cure rates.

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