Journal of Advanced Veterinary Research Volume 8, Issue 4 (2018) 95-100

Journal of Advanced Veterinary Research

http://advetresearch.com/index.php/avr/index

Detection of Methicillin-resistant and Biofilm-producing *Staphylococcus aureus* in Bovine Mastitis

Maha I. Hamed

Animal Medicine Department (Infectious Diseases)- Faculty of Veterinary Medicine- Assiut University- Assiut- Egypt, 71526

ARTICLE INFO

Original Research

Received:
09 August 2018

Accepted:
29 September 2018

Keywords:

Antibiotic resistance
Biofilm
Mastitis
S. aureus
MRSA

ABSTRACT

The present study was employed to investigate the causative microorganisms responsible for clinical and subclinical mastitis and their antibiotic susceptibility pattern on a private cow farm in Assiut Governorate, Egypt, where a history of recurrent mastitis was the main complaint. In addition, the isolates were investigated for their ability to form biofilm. Forty cows were subjected to a physical examination of the udder and milk using the California mastitis test. Additionally, milk sample collections were obtained, and bacterial specimens were isolated and evaluated for the presence of the *mecA* gene by PCR. Antibacterial susceptibility assays against the common antibiotics used in the veterinary field were conducted for all isolates obtained. Furthermore, biofilm production by bacterial isolates was detected using the microtiter plate method and the activity of specific antibiotics was evaluated against preformed biofilms. About 10% of the examined cows showed clinical signs of mastitis, and 22.2% revealed subclinical mastitis infection when evaluated via the California mastitis test. *Staphylococcus aureus* was isolated from all mastitic cows, which indicated that it was the main causative organism for the infection. *Streptococcus spp*. were isolated from six mastitic animals. Half of the *S. aureus* isolates were methicillin-resistant, and 83.33% of them were capable of producing biofilm. All *Streptococcus spp*. isolates were sensitive to all the antibiotics evaluated in the study. *S. aureus* isolates in the planktonic form were resistant to oxytetracycline and penicillin. In contrast, *S. aureus* encased in biofilm were resistant to all the antibiotics used in the study. This research detected highly-virulent *S. aureus* isolates from clinical and subclinical mastitic cases that carry the *mecA* gene and produce biofilm. The owner is advised to cull the diseased cows to prevent the spread of these virulent isolates to healthy animals. Also, it is advisable not to treat the diseased cows with the antibiotics evaluated in this study as they were found to be ineffective and may potentially contribute to persistence of the infection.

J. Adv. Vet. Res. (2018), 8 (4),95-100

Introduction

Staphylococcus aureus (*S. aureus*) has been identified as a major source of bovine clinical and subclinical mastitis (Szweda *et al*., 2012; Cervinkova *et al*., 2013). In recent years, methicillin-resistant *S. aureus* (MRSA) strains have been increasingly reported as an emerging crisis in bovine mastitis cases making this disease very challenging to treat (Vanderhaeghen *et al*., 2010; Saini *et al*., 2012; Zutic *et al*., 2012). Furthermore, cows with subclinical mastitis may act as a source of infection for other animals, resulting in the spread of mastitis infection to the rest of the herd (Sharma *et al*., 2015). Sayed (2014) investigated the problem of clinical mastitis in dairy cows within the Assiut Governorate, Egypt and found that *S. aureus* was the main causative agent (34.65%) with 60% of these isolates identified as MRSA. To ensure suitable antibiotic treatment, bacterial isolation and evaluation of antibiotic susceptibility are necessary. Early and specific antibiotic treatment can reduce the severity of the disease. However, extensive use of antibiotics over the years has contributed to an increase in antibiotic resistance. Although *S. aureus* is susceptible to a variety of antibiotics in vitro, treatment of intra-mammary infections results in a low cure rate, regardless of the antibiotic used (Keefe, 2012). This may be a result of incomplete penetration of the antibiotics throughout the gland and the potential survival of bacteria within host cells, leading to a recurrence of mastitis once treatment has ended (Hebert *et al*., 2000; Kerr *et al*., 2001; Barcia-Macay *et al*., 2006). Another contributing factor to the recurrence of mastitis may be due to the formation of biofilms (slime) that are resistant to the effect of many antibiotics (Cramton *et al*., 1999). The majority of *S. aureus* strains causing mastitis are surrounded by a slime layer, which helps bacteria to attach and colonize the mammary gland tissue (Baselga *et al*., 1993; Aguilar *et al*., 2001).

*Corresponding author: Maha Ibrahim Hamed
E-mail address: mahohzero@gmail.com

ISSN: 2090-6277/2090-6269/

The ability of *S. aureus* to bind to epithelial surfaces is associated with the production of a biofilm composed of multilayered cell clusters embedded in a slime matrix (Cucarella *et al*., 2001). Biofilm formation is associated with antimicrobial resistance because of the difficulty antibiotics have in diffusing through the polysaccharide matrix and reduced metabolic activity of bacteria within the biofilm (Amorena *et al*., 1999; Melchior *et al*., 2006; Yazdani *et al*., 2006). In Egypt, research on bacterial biofilms has only begun recently and mainly focused on *S. aureus* isolates from human sources (Gad *et al*., 2009; Nasr *et al*., 2012). The present study was designed to investigate the causative microorganisms responsible of bovine mastitis and their antibiotic susceptibility pattern on a private cow farm in Assiut Governorate, Egypt. The owner of the farm complained of recurrent mastitis in his herd that did not respond to different treatment options. One of the most common causes of failure in therapy is the ability of the causative bacteria to grow in biofilm. So, the study was aimed at also investigating the biofilm-forming ability of bacterial isolates that were the source of mastitis in these cows.

Materials and methods

Examination for mastitis

The study was conducted on a private cow farm (40 cows in total) within Assiut Governorate, Egypt, where a history of recurrent clinical mastitis in cows was the chief complaint. Some cases of mastitis responded to treatment with Tetra-Delta intra-mammary infusion (procaine penicillin and neomycin) for a few days before signs of mastitis reappeared again. In certain cases, diseased cows did not respond to the treatment at all. The cases of clinical mastitis were diagnosed based on history, clinical signs, and physical examination of udder and milk. Cases of subclinical mastitis were diagnosed using the California mastitis test (Schalm *et al*., 1971).

Sample collection and bacterial isolation

Milk samples were collected from all quarters of cows with clinical mastitis and subclinical mastitis (as identified by the California mastitis test). Udders of cows presenting with mastitis were washed with clean water and dried. Cotton swabs soaked with 70% ethanol were used to disinfect the surfaces of teats. The first few streams of milk were discarded before a milk sample was collected into a 10-ml sterile plastic tube. Samples were sent directly to the laboratory in an ice tank and cultured on the same day of sample collection. Milk samples were inoculated on 5% sheep blood agar plates and incubated at 37ºC for 24 h. *S. aureus* identification was confirmed via gram staining, colony morphology, and traditional biochemical tests, including catalase, coagulase and mannitol fermentation tests (Turk and Porter, 1978; Bannerman, 2003). Other pathogens were identified using laboratory procedures that followed the guidelines defined by the National Mastitis Council (NMC, 1999).

Detection of mecA gene

DNAs from *S. aureus* isolates were extracted using the DNAeasy Tissue Kit (QIAamp® DNA Mini Kit) with some modifications to the manufacturer's protocol. The extracted DNA was used as a template for PCR amplification. PCR amplifications were performed with a pair of primers specific for the *mecA* gene, synthesized from the previously published sequences: primer 1: 5'-AAA ATC GAT GGT AAA GGT TGG C-3', primer 2: 5'-AGT TCT GCA GTA CCG GAT TTG C-3' (Louie *et al*., 2002). The PCR cycles consisted of initial denaturation at 96°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30s, annealing at 60°C for 30s, and extension at 72°C for 1 min, and final extension at 72°C for 10 min. The PCR products were analyzed by electrophoresis in a 1.5% agarose gel containing 0.5 mg of ethidium bromide per ml. The sizes of the amplification products were estimated by comparison with a 100bp DNA step ladder.

Antibacterial assays

The selected antimicrobial agents (oxytetracycline, penicillin, clindamycin, ciprofloxacin, marbofloxacin, levofloxacin, vancomycin, ceftriaxone and enrofloxacin) were provided as powders by VWR, USA. The antimicrobial agents were subsequently dissolved in suitable solvents to make stock 10µg/ml solutions. The minimum inhibitory concentration (MIC) of each antibiotic was evaluated using the broth microdilution technique, in Mueller-Hinton broth (MHB), in accordance with the Clinical and Laboratory Standards Institute's (CLSI) guidelines (CLSI, 2007). The MIC was interpreted as the lowest concentration of antibiotic that completely inhibited the growth of bacteria after 16 h incubation of the plates at 37°C. Each agent was tested in triplicate in at least two independent experiments. The highest MIC value for each test agent was reported.

Detection of biofilm by Microtiter plate method (MTP)

The ability of *S. aureus* isolates to form biofilm was investigated by a method described by Stepanovic *et al*. (2007) with some modifications. Bacteria were cultivated overnight in Tryptic Soy Broth (TBS) supplemented with 1% glucose. Each culture was diluted 1:100 in the same medium and, subsequently, 200 µl of the diluted bacterial suspension was transferred, in triplicates, into the wells of sterile 96-well polystyrene microtiter plates and incubated at 37°C for 24 h. The negative control contained only the growth medium. The plates were washed twice with 200 µl of PBS (pH 7.2) and dried at room temperature prior to staining biofilms with 1% crystal violet. The plates were incubated at room temperature for 15 min before excess dye was removed by washing with water. The biofilm-bound dye was dissolved in 150 µl of 95% ethanol (SIGMA-ALDRICH Ethanol Lot # SZBE1180V Germany UN1170). The OD of dye (representing biofilm density) was measured at 595-nm by using a microplate reader (Spectra Max i3x, 363701003).

Antibiotic activity against preformed biofilms

The activity of antibiotics against 24 h-old biofilms was assessed by viable colony count. Biofilms were allowed to form in each well of a 96-well flat-bottom polystyrene tissue culture-treated microtiter plate (Becton, Dickinson and Company), as described above. Following 24 h-incubation, biofilms were washed twice with PBS, then exposed to 200 µl of drug-containing cation-adjusted Mueller–Hinton broth (CAMHB) (prepared with 1, 2, 4, 8, 16, 32, 64, and 128 × MIC of antibiotics). After incubation at 37 °C for 24 h, non-adherent bacteria were removed by washing twice with 200 µl sterile PBS, and biofilm samples were scraped as described above. The cell suspension was then vortexed for 1 min to break up bacterial clumps. Bacterial counts were performed by plating serial 10-fold dilutions of this suspension on MHA plates. Control biofilm samples were not exposed to antibiotics. The Minimum Biofilm Eradication Concentration (MBEC) was categorized as the minimum concentration of tested antibiotic able to completely eradicate mature biofilm.

Results

Examination results

Physical examination of the udder and milk identified four cows out of 40 (10%) with clinical mastitis, which were unsuccessfully treated with Tetra-Delta intra-mammary infusion. Examination of clinically healthy cows (n.=36) using the California mastitis test revealed eight cows with subclinical mastitis (22.2%).

Bacteriology results

S. aureus was isolated from all 12 mastitic milk samples. Furthermore, *Streptococcus spp*. were isolated from six mastitic milk samples only (all four samples from clinical mastitic cows and two samples from subclinical mastitic cows).

mecA gene detection results

Of the 12 *S. aureus* isolates obtained from the mastitic cows, six isolates were *mecA* gene positive (MRSA) and the other six isolates were negative. The PCR product of MRSA isolates revealed a positive band at 533bp on the agarose gel electrophoresis (Fig. 1).

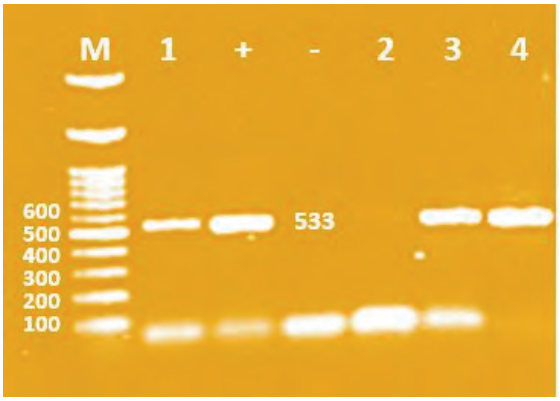

Fig. 1. Agarose gel showing PCR products of *mecA* positive *S. aureus* isolates from mastitis cases (positive band at 533 bp). M = Marker, lane (+) = positive control, lane (-) = negative control, lane (1,3,4) = positive samples and lane (2) = negative sample.

Antibacterial assay results

The *Streptococcus spp*. isolates were sensitive to all the antibiotic evaluated via the broth microdilution assay. *S. aureus* isolates in the planktonic form were also sensitive to all antibiotics used in the assay except for oxytetracycline and penicillin (Table 1).

Detection of biofilm results

All six MRSA isolates were capable of producing a biofilm on the 96-well polystyrene microtiter plates. Meanwhile, only four isolates of *mecA* negative *S. aureus* could form a biofilm. Thus, 10 out of 12 *S. aureus* isolates were found to be capable of forming a biofilm. As shown in Fig. 2, crystal violet dye bound to the bacteria adhered to the bottom of the wells indicating biofilm formation.

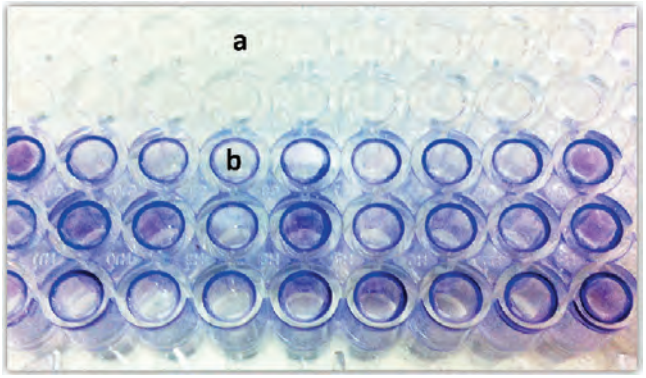

Fig. 2. Microtiter plates showing *S. aureus* biofilm producing isolates differentiated by crystal violet staining in 96-well tissue culture plates, a. negative and b. wells with biofilm.

Results of antibiotic activity against preformed biofilms

The MBEC of antibiotics against *S. aureus* and MRSA isolates ranged from 8 to >128 µg/ml, which indicates that bacteria present within the biofilm are resistant to the antibiotics evaluated (Table 2).

Table 1. Antimicrobial susceptibility pattern of *Streptococcus spp*. and *S. aureus* isolated from mastitic milk samples using the broth microdilution technique.

Antibiotics	MIC Range (µg/ml)	
	Streptococcus spp. (6 isolates)	*S. aureus* (12 isolates)
Oxytetracycline	0.25	0.125 – 4 (4 isolates) 16 – 64 (8 isolates)
Penicillin	2 – 8	0.25 – 2 (4 isolates) 16 – 32 (8 isolates)
Vancomycin	0.125 – 1	0.5 – 8
Ciprofloxacin	2	0.0625 – 0.5
Clindamycin	0.125	<0.0156 – 0.5
Ceftriaxone	0.5	0.125 – 4
Levofloxacin	1	0.125 – 4
Enrofloxacin	0.5	0.125 – 0.5
Marbofloxacin	1 – 2	0.25 – 2

Table 2. In vitro antimicrobial susceptibility test of the biofilm forming *S. aureus* isolates in planktonic and biofilm form using the broth microdilution technique.

Isolates	(µg/ml)													
	Oxytetracycline		Penicillin		Vancomycin		Ciprofloxacin		Clindamycin		Ceftriaxone		Levofloxacin		Enrofloxacin		Marbofloxacin	
	MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC	MBEC
S. aureus (4 isolates)	16	>128	32	>128	4	8	0.5	16	0.125	32	4	>128	0.5	16	0.25	16	0.5	16
MRSA (6 isolates)	32	>128	16	>128	8	16	0.5	32	0.125	64	2	>128	0.25	64	0.25	64	0.25	32

Discussion

Mastitis affects dairy cows on every dairy farm in Egypt. In particular, contagious mastitis caused by *S. aureus*, is a costly and critical problem in the dairy industry. Treatment and eradication of *S. aureus* in mastitis cases is difficult, because of the frequent emergence of bacterial resistance to antimicrobials. Recently, an increasing rate of antimicrobial resistance has been identified in *S. aureus* strains isolated from bovine mastitis (Saini *et al*., 2012; Wang *et al*., 2013). This is generally attributed to misuse and/or continuous use of antibacterial drugs without prior drug susceptibility testing or to the colonization of the mammary gland by resistant strains. These antimicrobial resistant organisms can induce serious health problems to both animals and human beings.

In the present study of 40 cows on one dairy farm in Egypt, *S. aureus* was isolated from all 12 cows presenting with mastitis. Meanwhile, *Streptococcus spp*. was isolated only from four cases, which indicates that *S. aureus* is the main causative pathogen of mastitis in the examined cows. This is in agreement with other published reports (Sumathi *et al*., 2008; Singh *et al*., 2016) that found a higher prevalence of Staphylococcus spp. in the milk of cows with mastitis. Moreover, Moroni *et al*. (2006) stated in their report that *S. aureus* was frequently isolated in bovine clinical and subclinical mastitis.

Half (50%) of the *S. aureus* strains isolated from mastitic milk in this study were *mecA* positive (MRSA), which indicates a high prevalence of MRSA strains among mastitic cows. This finding coincided with Zutic *et al*. (2012) who indicated that methicillin resistance is widely spread among *S. aureus* isolates from bovine milk. Sharma *et al*. (2015) also found a high prevalence of MRSA among bovines used for milk production in India.

S. aureus isolates in the planktonic form were sensitive to all the antibiotics used in the study, except for oxytetracycline and penicillin. Similar results were reported for *S. aureus* isolated from the milk of cows by Rajala-Schultz *et al*. (2004); Wang *et al*. (2008); Coelho *et al*. (2009) and Bhatt *et al*. (2011). They indicated that β-lactams (penicillins) and tetracycline are widely used for intra-mammary treatment of staphylococcal bovine mastitis. Not surprisingly then, these drugs are most frequently associated with antimicrobial resistance. Costa *et al*. (2000) also confirmed that *S. aureus* resistance to penicillin and/or ampicillin was frequently observed (44.5%) in isolates from both cows and heifers. Our results found *S. aureus* isolates exhibiting high sensitivity to fluoroquinolones specially enrofloxacin, which indicate these drugs may play an important role for treatment of *S. aureus* infections in veterinary medicine (Beco *et al*., 2013).

After evaluating the antibiotic susceptibility profile of *S. aureus* isolates obtained from cows in our study, we next investigated the isolates' ability to form biofilms. The MTP method was employed as it has been reported to have high specificity, sensitivity, and positive predictive values (Mathur *et al*., 2006). Results of this study indicated that 10 (83.33%) out of the 12 *S. aureus* isolates we investigated were capable of forming biofilm using the MTP method. This finding was in agreement with Darwish and Asfour (2013) who found that 96.3% of Staphylococcus spp. isolates were capable of forming biofilms using the MTP method. Similarly, Schönborn *et al*. (2017) found that 74.2% of *S. aureus* strains that caused mastitis were capable of producing biofilm. The remaining two isolates from our study that did not form a biofilm, could be due to the growth conditions used. *S. aureus* biofilm formation is highly-sensitive to growth conditions, such as the amount of glucose available for matrix formation (Cramton *et al*., 1999). In addition, some capsular exopolysaccharides are not well expressed in the presence of oxygen (Gotz, 2002).

After confirming the ability of ten *S. aureus* isolates to form biofilm, we investigated the susceptibility of these biofilms to nine different antibiotics. All *S. aureus* isolates present within the biofilm were resistant to all the antibiotics used in this study. This most likely is an important factor that has contributed to chronic and recurrent mastitis among the farm cows that are resistant to antibiotic treatment. The ability of *S. aureus* to form biofilms helps the bacterium to survive within the host and is responsible for chronic or persistent infections (Costerton *et al*., 1999). Biofilms facilitate the adherence and colonization of bacteria onto the mammary gland epithelium which leads to difficulty in eradicating the pathogen (Zadoks *et al*., 2002; Vasudevan *et al*., 2003; Cucarella *et al*., 2004; Brouillette *et al*., 2005; Fox *et al*., 2005; Melchior *et al*., 2006). Several studies showed that bacteria in biofilms are 10 to 1000 times more resistant to the effects of antibiotics as compared to the same strain in the planktonic form (Amorena *et al*., 1999; Ceri *et al*., 1999; Mah and O'Toole, 2001; Olson *et al*., 2002; Conley *et al*., 2003). The production of an exopolysaccharide matrix is one of the important characteristics of biofilms. This matrix acts like a capsule that impairs the access of antibiotics to bacterial cells (Stewart, 1996). The slow growth of the bacteria in biofilms is also responsible for the decreased susceptibility of bacteria to antibiotics that require organisms to be actively growing/dividing. For example, penicillins and cephalosporins are ineffective on non-growing cells, and the rate of bacterial killing is proportional to the growth rate (Costerton *et al*., 1999). Some antibiotics like tetracyclines and erythromycin are not only less effective against bacterial biofilms but may actually stimulate biofilm formation (Melchior *et al*., 2006). Primary bacterial infections usually consist of the invasive planktonic form of the bacteria, whereas in chronic infections biofilms may be involved. Thus, it is important to start early treatment when a rise in somatic cell count indicates an intra-mammary subclinical infection (De Haas *et al*., 2002). Early onset of treatment as well as prolongation of the treatment period both can be expected to improve cure rates (Melchior *et al.*, 2006).

Conclusion

This study found that *S. aureus* isolated from 12 bovine clinical and subclinical mastitis cases carried the *mecA* gene and were capable of producing biofilm. Moreover, isolates capable of forming biofilms were found to be resistant to most of the antibiotics used in veterinary practice. Therefore, treating mastitic cows with antibiotics, on this particular farm, was not a viable choice, and culling of these cases was suggested as the best treatment option to protect the rest of the herd. Treatment failure most likely was due to the formation of bacterial biofilms. Biofilms are difficult to eradicate, so in the future the focus must be directed toward effective prophylaxis and developing a suitable treatment regime.

Acknowledgement

I would like to thank Dr. Mohamed Seleem of Purdue University for providing the antibiotics and some of the equipment used in this study. Also, I would like to thank Dr. Haroon Mohamed of Purdue University for editing the manuscript.

References

Aguilar, B., Amorena, B., Iturralde, M., 2001. Effect of slime on adherence of *Staphylococcus aureus* isolated from bovine and ovine mastitis. Vet. Microbiol. 78,183–191.

Amorena, B., Gracia, E., Monzon, M., Leiva, J., Oteiza, C., Perez, J.L., Alabart, J.L., Hernandez-Yago, J., 1999. Antibiotic susceptibility assay for *Staphylococcus aureus* in biofilms developed in vitro. Journal of Antimicrobial Chemotherapy 44, 43–55.

Bannerman, T.L., 2003. Staphylococcus, Micrococcus, and other catalase-positive cocci that grow aerobically, p. 384-404. In Murray PR, Baron EJ, Jorgensen JH, *et al.* (ed.). Manual of Clinical Microbiology. 8th ed. vol. 1. American Society for Microbiology, Washington, D.C.

Barcia-Macay, M., Seral, C., Mingeot-Leclercq, M.P., Tulkens, P.M., Van Bambeke, F., 2006. Pharmacodynamic evaluation of the intracellular activities of antibiotics against *Staphylococcus aureus* in a model of THP-1 macrophages. Antimicrob Agents Chemother. 50, 841-851.

Baselga, R., Albizu, I., De La Cruz, M., Del Cacho, E., Barberan, M., Amorena, B., 1993. Phase variation of slime production in *Staphylococcus aureus*: implications in colonization and virulence. Infection and Immunity 61, 4857–4862.

Beco, L., Guaguère, E., Lorente Méndez, C., Noli, C., Nuttall, T., Vroom, M., 2013. Suggested guidelines for using systemic antimicrobials in bacterial skin infections: part 2-antimicrobial choice, treatment regimens and compliance. Vet. Rec. 172, 156–160.

Bhatt, V.D., Patel, M.S., Joshi, C.G., Kunjadia A., 2011. Identification and antibiogram of microbes associated with bovine mastitis. Anim Biotechnol. 22, 163-169.

Brouillette, E., Hyodo, M., Hayakawa, Y., Karaolis, D.K.R., Malouin, F., 2005. 30,50-Cyclic diguanylic acid reduces the virulence of biofilm-forming *Staphylococcus aureus* strains in a mouse model of mastitis infection. Antimicrob Agents Chemother 49, 3109–3113.

Ceri, H., Olson, M.E., Stremick, C., Read, R.R., Morck, D., Buret, A., 1999. The calgary biofilm device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. Journal of Clinical Microbiology 37, 1771–1776.

Cervinkova, D., Vlkova, H., Borodacova, I., Makovcova, J., Babak, V., Lorencova, A., Vrtkova, I., Marosevic, D., Jaglic, Z., 2013. Prevalence of mastitis pathogens in milk from clinically healthy cows. Veterinarni Medicina 58, 567-575.

CLSI, 2007. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard M7-A7. CLSI, Wayne, PA.

Coelho, S.M.O., Reinoso, E., Pereira, I.A., Soares, L.C., Demo, M., Bogni, C., Souza, M.M.S., 2009. Virulence factors and antimicrobial resistance of *Staphylococcus aureus* isolated from bovine mastitis in Rio de Janeiro. Pesquisa Veterinária Brassileira 29, 369–374.

Conley, J., Olson, M.E., Cook, L.S., Ceri, H., Phan, V., Davies, H.D., 2003. Biofilm formation by group A streptococci: is there a relationship with treatment failure? Journal of Clinical Microbiology 41, 4043–4048.

Costa, E.O., Benites, N.R., Guerra, J.L., Melville, P.A., 2000. Antimicrobial susceptibility of Staphylococcus spp. isolated from mammary parenchymas of slaughtered dairy cows. Journal of Veterinary Medicine. B, Infectious Diseases and Veterinary Public Health 47, 99–103.

Costerton, J.W., Stewart, P.S., Greenberg, E.P., 1999. Bacterial biofilms: a common cause of persistent infection. Science 284,1318–1322.

Cramton, S.E., Gerke, C., Schnell, N.F., Nichols, W.W., Götz, F., 1999. The intercellular adhesion (ica) locus is present in *Staphylococcus aureus* and is required for biofilm formation. Infection and Immunity 67(10), 5427–5433.

Cucarella, C., ÁngelesTormo, M., Ubeda, C., Trotonda, M.P., Monzón, M., Peris, C., Amorena, B., Lasa, I., Penadés, J.R., 2004. Role of biofilm associated protein Bap in the pathogenesis of bovine *Staphylococcus aureus*. Infect. Immun. 72, 2177–2185.

Cucarella, C., Solano, C., Valle, J., Amorena, B., Lasa, I., Penadés, J.R., 2001. Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. Journal of Bacteriology 183(9), 2888–2896.

Darwish, S.F., Asfour, H.A.E., 2013. Investigation of Biofilm Forming Ability in Staphylococci Causing Bovine Mastitis Using Phenotypic and Genotypic Assays. The Scientific World Journal. Article ID 378492, 9 pages. http://dx.doi.org/10.1155/2013/378492

De Haas, Y., Barkema, H.W., Veerkamp, R.F., 2002. The effect of pathogen-specific clinical mastitis on the lactation curve for somatic cell count. Journal of Dairy Science 85, 1314–1323.

Fox, L.K., Zadoks, R.N., Gaskins, C.T., 2005. Biofilm production by *Staphylococcus aureus* associated with intramammary infec-

tion. Vet. Microbiol. 107, 295–299.
Gad, G.F.M., El-Feky, M.A., El-Rehewy, M.S., Hassan, M.A., Abolella, H., El-Baky, R.M., 2009. Detection of icaA, icaD genes and biofilm production by *Staphylococcus aureus* and *Staphylococcus epidermidis* isolated from urinary tract catheterized patients. Journal of Infection in Developing Countries 3(5), 342–351.
Gotz, F., 2002. Micro review on Staphylococcus and biofilms. Molecular Microbiology 43, 1367–1378.
Hebert, A., Sayasith, K., Senechal, S., Dubreuil, P., Lagacé, J., 2000. Demonstration of intracellular *Staphylococcus aureus* in bovine mastitis alveolar cells and macrophages isolated from naturally infected cow milk. FEMS Microbiol Lett 193,57-62.
Keefe, G., 2012. Update on Control of *Staphylococcus aureus* and *Streptococcus agalactiae* for Management of Mastitis. Vet. Clin. N. Am-Food A 28,203-216.
Kerr, D.E., Plaut, K., Bramley, A.J., Williamson, C.M., Lax, A.J., Moore, K., Wells, K.D., Wall, R.J., 2001. Lysostaphin expression in mammary glands confers protection against staphylococcal infection in transgenic mice. Nat. Biotechnol. 19, 66-70.
Louie, L., Goodfellow, J., Mathieu, P., Glatt, A., Louie, M., Simor, A.E., 2002. Rapid detection of methicillin-resistant staphylococci from blood culture bottles by using a multiplex PCR assay. J. Clin. Microbiol. 40, 2786–2790.
Mah, T.F., O'Toole, G.A., 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends in Microbiology 9, 34–39.
Mathur, T., Singhal, S., Khan, S., Upadhyay, D.J., Fatma, T., Rattan, A., 2006. Detection of biofilm formation among the clinical isolates of staphylococci: an evaluation of three different screening methods. Indian Journal of Medical Microbiology 24(1), 25–29.
Melchior, M.B., Vaarkamp, H., Gremmels, J.F., 2006. Biofilms: a role in recurrent mastitis infections? The Veterinary Journal 171, 398–407.
Moroni, P., Pisoni, G., Antonini, M., Villa, R., Boettcher, P., Carli, S., 2006. Short communication: Antimicrobial drug susceptibility of *Staphylococcus aureus* from subclinical bovine mastitis in Italy. J. Dairy Sci. 89, 2973-6.
Nasr, R.A., AbuShadady, H.M., Hussein, S.H., 2012. Biofilm formation and presence of icaAD gene in clinical isolates of staphylococci. The Egyptian Journal of Medical Human Genetics 13, 269–274.
National Mastitis Council (NMC), 1999. Laboratory Handbook on Bovine Mastitis (Revised Edition). National Mastitis Council Inc., Madison, pp. 64–83.
Olson, M.E., Ceri, H., Morck, D.W., Buret, A.G., Read, R.R., 2002. Biofilm bacteria: formation and comparative susceptibility to antibiotics. Canadian Journal of Veterinary Research 66, 86–92.
Rajala-Schultz, P.J., Smith, K.L., Hogan, J.S., Love, B.C., 2004. Antimicrobial susceptibility of mastitis pathogens from first lactation and older cows. Vet. Microbiol. 102, 33-42.
Saini, V., McClure, J.T., Leger, D., Keefe, G.P., Scholl, D.T., Morck, D.W., Barkema, H.W., 2012. Antimicrobial resistance profiles of common mastitis pathogens on Canadian dairy farms. J. Dairy Sci. 95, 4319-4332.
Sayed, S.M., 2014. Bacteriological Study on Staphylococcal Bovine Clinical Mastitis with Reference to Methicillin-Resistant Staph. aureus (MRSA). Assiut Vet. Med. J. 60, 38-46.
Schalm, O.W., Carroll, G., Jain, N.C., 1971. Bovine Mastitis. Lea and Fibiger. Philadelphia. USA, 360.
Schönborn, S., Wente, N., Paduchand, J., Krömker, V., 2017. In vitro ability of mastitis causing pathogens to form biofilms. Journal of Dairy Research 84, 198–201.
Sharma, L., Verma, A.K., Kumar, A., Rahat A., Neha, Nigam, R., 2015. Incidence and Pattern of Antibiotic Resistance of *Staphylococcus aureus* Isolated from Clinical and Subclinical Mastitis in Cattle and Buffaloes. Asian Journal of Anmal Sciences 9 (3), 100-109.
Singh, N., Singh, P., Patel, R.K., 2016. Isolation and identification of bacterial organisms from mastitic milk. J. Livestock Sci. 7, 46-8.
Stepanovic, S., Vukovic, D., Hola, V., Di Bonaventura, G., Djukić, S., Cirković, I., Ruzicka, F., 2007. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. APMIS 115, 891-9.
Stewart, P.S., 1996. Theoretical aspects of antibiotic diffusion into microbial biofilms. Antimicrob. Agents Chemother. 40(11), 2517–2522.
Sumathi, B.R., Veeregowda, B.M., Amitha, R.G., 2008. Prevalence and antibiogram profile of bacterial isolates from clinical bovine mastitis. Vet. World 1, 237-238.
Szweda, P., Schielmann, M., Milewski, S., Frankowska, A., Jakubczak, A., 2012. Biofilm Production and Presence of ica and bap Genes in *Staphylococcus aureus* Strains Isolated from Cows with Mastitis in the Eastern Poland. Journal of Microbiology 61, 65- 69.
Turk, D.C., Porter, I.A., 1978. A short textbook of medical microbiology. 4th ed. London: Hodder and Stoughton.
Vanderhaeghen, W., Cerpentier, T., Adriaensen, C., Vicca, J., Hermans, K., Butaye, P., 2010. Methicillin-resistant *Staphylococcus aureus* (MRSA) ST398 associated with clinical and subclinical mastitis in Belgian cows. Vet. Microbiol. 144,166-171.
Vasudevan, P., Nair, M.K., Annamalai, T., Venkitanarayanan, K.S., 2003. Phenotypic and genotypic characterization of bovine mastitis isolates of *Staphylococcus aureus* for biofilm formation. Veterinary Microbiology 92, 179–185.
Wang, S., Wu, C., Shen, J., Wu, Y., Wang, Y., 2013. Hypermutable *Staphylococcus aureus* strains present at high frequency in subclinical bovine mastitis isolates are associated with the development of antibiotic resistance. Vet. Microbiol.165, 410-415.
Wang, Y., Wu, C.M., Lu, L.M., Ren, G.W., Cao, X.Y., Shen, J.Z., 2008. Macrolide–lincosamide- resistant phenotypes and genotypes of *Staphylococcus aureus* isolated from bovine clinical mastitis. Vet. Microbiol. 130, 118-25.
Yazdani, R., Oshaghi, M., Havayi, A., Pishva, A., Salehi, R., Sadeghizadeh, M., Foroohes, H., 2006. Detection of icaAD gene and biofilm formation in *Staphylococcus aureus* isolates from wound infection. Iranian J. Publ. Health 35, 25–28.
Zadoks, R.N., van Leeuwen, W.B., Kreft, D., Fox, L.K., Barkema, H.W., Schukken, Y.H., van Belkum, A., 2002. Comparison of *Staphylococcus aureus* isolates from bovine and human skin, milking equipment, and bovine milk by phage typing, pulse-field gel electrophoresis, and binary typing. J. Clin. Microbiol. 40, 3894–3902.
Zutic, M., Cirkovic, I., Pavlovic, L., Zutic, J., Asanin, J, Radanovic, O., Pavlovic, N., 2012. Occurrence of methicillin-resistant *Staphylococcus aureus* in milk samples from Serbian cows with subclinical mastitis. Afr. J. Microbiol. Res. 6, 5887-5889.