Short Communication: Antibacterial activity of Cefquinome Sulfate in the presence of biofilm *Staphylococcus* spp isolated from bovine milk

Comunicação breve: Actividade antibacteriana do sulfato de Cefquinome na presença do biofilme Staphylococcus spp isolado do leite bovino

DOI:10.34117/bjdv7n7-144
Recebimento dos originais: 07/06/2021
Aceitação para publicação: 02/07/2021

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ABSTRACT
Bovine mastitis is an inflammation of the mammary gland in dairy cattle usually caused by bacterial agents and cefquinome is a fourth-generation cephalosporin commonly used in treatment of this disease caused by *Staphylococcus* spp. in lactating cows due to a broad spectrum. On the other hand, biofilm formation provides protection for bacteria increasing the resistance to antibiotics and contributing to the persistence of infection. The objective of the present study was to assess if biofilm produced by *Staphylococcus* spp was able to protect the bacteria from cefquinome sulfate (CFQ) action for 24h exposure. Our findings showed that 95% (n=53/56) of *Staphylococcus* spp. were biofilm producer and the biofilm protected the most of species tested, except *S. xylosus* maybe due to the formation of a thin layer biofilm that did not interfere in the antimicrobial action. Thus, the effectiveness of cefquinome in persistent infections may be compromised due to a thicker biofilm.

Keywords: Biofilm, Mastitis, Resistance, *Staphylococcus* spp.

ABSTRACT
A mastite bovina é uma inflamação da glândula mamária em bovinos leiteiros causada geralmente por agentes bacterianos e o cefquinoma é uma cefalosporina de quarta geração geralmente utilizada no tratamento desta doença causada por Staphylococcus spp. em vacas em lactação devido a um largo espectro. Por outro lado, a formação de biofilme proporciona protecção às bactérias aumentando a resistência aos antibióticos e contribuindo para a persistência da infecção. O objectivo do presente estudo era avaliar se o biofilme produzido por Staphylococcus spp era capaz de proteger as bactérias da acção do sulfato de cefquinoma (CFQ) durante 24h de exposição. Os nossos resultados mostraram que 95% (n=53/56) de Staphylococcus spp. eram produtores de biofilme e o biofilme protegia a maioria das espécies testadas, excepto S. xylosus, talvez devido à formação de um biofilme de camada fina que não interferia na acção antimicrobiana. Assim, a eficácia do cefquinoma em infecções persistentes pode ser comprometida devido a um biofilme mais espesso.

Palavras-chave: Biofilme, Mastite, Resistência, Staphylococcus spp.

1 SHORT COMMUNICATION

Bovine mastitis is an inflammation of the mammary gland in dairy cattle usually caused by bacterial agents. Among the main pathogens involved, the genus *Staphylococcus*, especially *Staphylococcus aureus* species, stands out. However, in recent years, the number of coagulase-negative staphylococci (CNS) isolates, previously considered only commensal bacteria, has increased surprisingly both in the clinical and subclinical context (Felipe et al., 2017). This is also due to the ability of these pathogens to produce biofilm in the udder epithelial cells of the cow, which blocks the action of antibiotics, ensuring persistence and bacterial proliferation (Goetz et al., 2017).

Antibiotic therapy is the most common treatment of bovine mastitis-infected dairy cows (Isaac et al., 2017). Cefquinome is a fourth-generation cephalosporin with antibacterial properties valuable in the treatment of mastitis in lactating cows due to a broad spectrum, and good activity against staphylococci species (Zonca et al., 2011). The objective of this study was to assess if biofilm of *Staphylococcus* spp was able to protect the bacteria from cefquinome sulfate (CFQ) action for 24h exposure.

Fifty six *Staphylococcus* spp strains isolated from milk cows with subclinical mastitis including, *S. aureus* (n=12), *S. epidermidis* (n=7), *S. saprophyticus* (n=10), *S. warneri* (n=15), *S. haemolyticus* (n=5), *S. xylosus* (n=3), *S. cohinii* (n=1), *S. capitis* (n=1) *and S. simulans* (n=2) were tested to verify the biofilm formation according to Stepanovic et al. (2000). The strains were collected from previous studies. All 56 samples were plated on blood agar and incubated at 35°/24h. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) was performed to confirm the species according to Barcelos et al. (2019). Isolates were grown in Brain Heart Infusion (BHI) incubated at 35°C/24 h and diluted using 0.5 Mc Farland scale with Densicheck (bioMeriéux, Marcy-l'Étoile, France). An aliquot of 200 µl was added in quadruplicate into 96-well microplate, incubated for 35°C/48h without shacking. After, the plate was washed three times with phosphate buffered saline (PBS, pH 7.2), dried at room temperature and stained with 1% crystal violet for 15 min. After three washes using the same buffer and drying at room temperature, plates were placed in an ELISA reader (Biotek, Winooski, VT) at 570 nm. Uninoculated BHI was used as a blank to correct the absorbance value, from an average of four wells. The strains were classified as non-producer, weak, moderate and strong producer according to Stepanovic et al. (2007).

In order to evaluate the antimicrobial susceptibility to Cefquinome sulfate (Sigma-Aldrich) one strain of each species was tested by the determination of minimum

bactericidal concentration (MBC) according to CLSI (2018) and the CFQ was dissolved and diluted in sterile distilled water. The isolates were incubated at 37°C for 24 h in BHI. The susceptibility to cefquinome was checked using a resazurin microtiter assay method at concentrations ranging from 2.45 to 0.0006 µg/mL and using a bacterial standardized suspension (10^5 CFU/mL). The microplate was incubated at 35°C for 24 h. After this, inocula with no visual growth were plated on TSA (T*ryptic Soy Agar*, *Difco)* and incubated at 35°C for 24 h. The MBC value was determined by the highest dilution that completely inhibited bacterial growth on TSA plates. After MBC determination, the biofilm assay was performed as described above, using only the producer isolates previously tested. Then, the wells were washed 4 times with sterile PBS (pH 7.2), 200 µL of cefquinome sulfate with a MBC value for each species (Table 1) was added, and the microplate was incubated at 35°C for 24 h. After this, all contents from the wells were plated onto TSA to confirm bactericidal efficiency. The wells were filled with sterile PBS, and the biofilm was broken using an ultrasonic processor (UP100H; Hielscher, Teltow, Germany; 5 cycles of 5 s each, 30% amplitude) and the contents were plated onto TSA to observe the presence or absence of growth after incubation at 35°C/24 h. The presence of growth after sonication confirms that the biofilm protected the bacterial cells below it.

The biofilm production occurred in 94.6% (N=53/56) being 83% (N=44/53) strong biofilm producer; 11.3% (N=6/53) moderate biofilm producer and 5.6% (N=3/53) weak biofilm producer. When analyzed the susceptibility of CFQ in presence of biofilm with MBC specific for each species, after 24h of incubation, was observed that all isolates, except *S. xylosus*, provided the same profile, with growth after sonication showing that the biofilm protected the strains. (Table1).

Table1**:** Cefquinome sulfate minimal bactericidal concentration, and biofilm production by *Staphylococcus* spp, isolated from milk of cows with subclinical mastitis.

Species	**No. of isolates**	**Biofilm Producer (n)**	**WP (OD)**	**MP (OD)**	**SP (OD)**	**MBC CFQ (µg/mL)**
S. warneri	15	13	-	1(0.27)	12 (1.2)	1.95
S.aureus	12	12	-	3 (0.36)	9 (1.2)	0.98
S.saprophyticus	10	10	-	-	10 (1.27)	0.98
S.epidermidis	7	6	-	1 (0.22)	5 (1.0)	1.95
S.haemolyticus	5	5	-	1 (0.27)	4 (1.7)	0.49
S. xylosus	3	3	3 (0.18)	-	-	15.62
S.simulans	2	2	-	-	2 (0.65)	0.98
S.cohinii	1	1	-	-	1 (1.33)	0.49
S.capitis	1	1	-	-	1 (0.73)	0.98

*WP:weak producer; MP:moderate producer; SP:strong producer; MBC: minimum bactericidal concentration; CFQ= cefquinome sulfate, OD: optical density.

Several types of antimicrobials can be used in the treatment of cows with mastitis caused by *Staphylococcus* spp. and CFQ has been approved for the treatment by the major mastitis-causing pathogens. Our results showed that antimicrobial had no effect on *Staphylococcus* spp. considered moderate or strong biofilm producer. Felipe et al. (2019) suggested that biofilms may resist to higher levels of antibiotics reducing their efficacy. *Staphylococcus xylosus* was the unique specie that no grown after sonication. According to Okuda et al. (2018), Staphylococcal strains can contribute differently for the composition of the extracellular matrix influencing in thickness biofilm so, we can suggest that low optical density of *S. xylosus* (Table 1) is due to the formation a thin layer of biofilm that did not interfere in the antimicrobial action. The limitation of this study is that we cannot to measure the real behavior of *S. xylosus* once we have only three strains and those were classified as weak biofilm producer. Our results disagree with Tremblay et al. (2013) that related *S. xylosus* as the species with the highest ability to form biofilm but, did not test the antimicrobial action. As far as we know, there are no studies reporting the influence of Cefquinome sulfate in polysaccharide matrix by *S. xylosus* nor with other *Staphylococcus* spp. Biofilm by *Staphylococcus* spp have been proposed as important factor in the persistence of intramammary infection. Thus, the effectiveness of cefquinome in persistent infections may be compromised according to thickness biofilm.

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