Biofilm expression and antimicrobial resistance patterns of *Streptococcus uberis* isolated from milk samples of dairy cows in South Africa

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Abstract
The research described in this Research Communication addresses the hypothesis that intramammary infections with *Streptococcus uberis* (*S. uberis*) are associated with biofilm formation, which limits antibiotic efficacy. This retrospective study investigated biofilm expression and antimicrobial resistance (AMR) patterns of 172 *S. uberis* infections. Isolates were recovered from milk samples of subclinical, clinical, and intramammary infection cases on 30 commercial dairy herds. We determined the presence and intensity of biofilm expression of *S. uberis* isolates in vitro in three somatic cell count categories to recognise their AMR patterns. An automated minimum inhibitory concentration system with a commercially available panel of 23 antimicrobial agents evaluated AMR, while biofilm determination was conducted using a microplate method. The study established that all the *S. uberis* isolates assessed expressed biofilm with the following varying degrees of intensities: 30 (17.8%) strong, 59 (34.9%) medium and 80 (47.3%) weak biofilms. The newly registered UBAC mastitis vaccine containing biofilm adhesion components may, therefore, be a viable option for proactive mastitis management under field conditions. No differences were identified between biofilm intensity and the three somatic cell count groups. Most *S. uberis* isolates indicated a highlevel sensitivity to the antimicrobial agents tested. Resistances were present in 8.7, 8.1 and 7.0% cases to rifampin, minocycline and tetracycline, respectively. Multidrug resistance was observed in 6.4%, emphasising AMR to antibiotics used in human medicine only. The low overall resistance suggests that farmers adhere to the prudent use of antimicrobials in the dairy industry.

Keywords:
Antimicrobial resistance; biofilm; dairy cows; somatic cell count; *Streptococcus uberis*

A diverse pathogen group can cause bovine mastitis. *Streptococcus uberis* is a predominant pathogen associated with subclinical and clinical mastitis (for this and additional statements see supporting literature in online Supplementary File). *S. uberis* is an intracellular and opportunistic pathogen that can adapt to and survive in various environments attributable to its nutritional flexibility. Although *S. uberis* is an environmental pathogen, its host-adapted strains can adhere to the mammary gland epithelial cells, causing persistent and recurrent infections. These bacteria can colonise multiple body sites, including the intestinal and genital tracts and the mammary gland.

A hyaluronic acid capsule, an extracellular virulence factor favours the ability of *S. uberis* to

survive in the environment. *S. uberis* is excreted in bovine faeces and can be present in bedding material and in dairy pastures. Because of the inflated cost of traditional bedding materials, using physically separated slurry or recycled manure solids as bedding material has become more pronounced in recent years in New Zealand, the Netherlands and South Africa (Blignaut et al., 2018; Klaas and Zadoks, 2018). This practice increases the risk of continuously inoculating environmental pathogens into grazing pastures. Host resistance, bacterial load and contact opportunities between pathogens and hosts remain crucial drivers of mastitis infection risk (Klaas and Zadoks, 2018).

Biofilm is an important virulence factor aiding bacteria in evading the udder immune defence (Moore, 2009). The milk somatic cell count (SCC) increases when a cow's immune cells are released into the milk to combat pathogenic bacteria. A South African databank (2018–2020) at the milk laboratory at the University of Pretoria (*n* = 303 895) directed that 16.9% of milk isolates from samples were major gram-positive bacteria, of which 47.4% were *S. uberis*. The milk SCC of 80.4% of these *S. uberis* isolates exceeded 200 000 cells/ml of milk (Inge-Marié Petzer, personal communication).

The β-lactams (penicillin and cephalosporin) became the first antimicrobial agents to treat bovine mastitis in some countries, including South Africa. Reports in New Zealand and Switzerland reveal reduced sensitivity or resistance to both classes of antibiotics. Resistance to penicillin, amoxicillin/clavulanic acid, ampicillin, erythromycin and clindamycin was demonstrated in streptococci for mastitis studies in Egypt, although not in Uruguay. In South Africa, humans consume 98% of all penicillins and streptomycins; 69% use other antibiotics, such as tetracycline, macrolide, cephalosporins, and quinolone (National Department of Health, 2018). This may cause penicillin resistance in humans (Bolukaoto et al., 2015).

Intramammary infections with *S. uberis* are associated with biofilm formation that limits antibiotic efficacy (Moore, 2009). Other virulence factors of *S. uberis*, such as activation genes, can transfer antibiotic resistance genes among biofilm microcommunity Members. This can lead to emerging AMR to mastitis pathogens. Preliminary treatment for a prolonged period could improve cure. Therapeutic failure of antimicrobial agents increases the risk of developing antibiotic resistance, one of the greatest threats to human and animal health (Dhingra et al., 2020). A limited number of intramammary antimicrobials are approved for treating bovine mastitis in South Africa, therefore, judicious use of these products is crucial.

This study aimed to investigate *S. uberis* isolates from milk samples of South African dairy herds for in vitro biofilm expression intensities from various SCC groups. The study also determined the AMR patterns of 23 SCC-related antimicrobials.

Materials and methods

A detailed account of all methodologies is provided in the online Supplementary File.

Data source

This retrospective study used *S. uberis* isolates from cow milk samples of routine herd udder health from 2018 to 2021. The isolates originated from 30 commercial dairy herds across

eight South African provinces; these include clinical and subclinical mastitis cases and intramammary infections. Classical microbiology phenotypic methods initially identified *S. uberis* isolates, confirmed by the MALDI-TOF MS (Bruker Daltonics, Bremen, Germany).

Experimental design

Biofilm expression testing was performed in vitro on 169 *S. uberis* isolates, allocated at random according to three SCC categories. The three SCC groups comprised 58 isolates from clinical mastitis cases; 60 isolates were from subclinical (SCC ≥ 300 000 cells/ml), and 51 isolates from intramammary infections with SCC < 300 000 cells/ml milk, respectively. An AMR investigation with minimum inhibitory concentration (MIC) levels was performed on the identical 169 isolates and an additional three *S. uberis* isolates, 172 isolates.

Biofilm formation detection

The biofilm expression potential of selected *S. uberis* isolates was investigated as described by Stepanović et al. (2007) with slight modifications (detailed in online Supplementary File). Colonies were collected from three sites on the culture plate and inoculated into 5ml of triptose serum broth and 10% glycerol broth (Thermofisher Scientific, Massachusetts, United States) and placed in a mechanical shaking incubator for 24 h at 37°C. All samples were assessed in triplicate with two replicates.

Polystyrene tissue culture-treated plates (96-well) (Sigma-Aldrich, Costar, USA) were filled with 200 µl of the diluted broth. Positive and negative controls were used. Plates were incubated for 48 h at 37°C, washed three times, and oven dried as described in the online Supplementary File. Biofilm expression was assessed using the modified crystal violet assay (Stepanović et al., 2007); absorbance was determined using a microplate reader.

Biofilm interpretation

The biofilm production was interpreted according to the criteria described by Stepanović et al. (2007). The OD (optical densities) was calculated as an average and subtracted from the cut-off value to obtain the final OD for each isolate. The two American type culture collection (ATCC) strains were used as positive controls. The optical density cut-off value (ODc) was the mean optical density of the negative (triptose serum broth only) control wells plus thrice its standard deviations (Stepanović et al., 2007).

Somatic cell counting

The milk sample SCC was determined by fluoro-opto-electronic means using a Fossomatic FC (Rhine Ruhr, Wendywood, South Africa).

Antimicrobial susceptibility testing

The *S. uberis* isolates were subjected to automated antimicrobial susceptibility testing using a commercially available panel of 23 antimicrobials, representing 13 antimicrobial groups according to the package insert (MICroSTREP plus Panel Type 6, Beckman Coulter). Results were evaluated according to the Clinical Laboratory Standards Institute (as described in online Supplementary file). The selected panel contained the antibiotics available as intramammary products in South Africa. Two *S. uberis* reference strains, ATCC 27958 and ATCC 700407 (Thermo Fisher Scientific, Massachusetts, United States) were used as controls. The susceptible breakpoints used for the MIC for each antibiotic were as stipulated

in the Clinical and Laboratory Standards Institute version VET01S-ED5:2020 and M100-ED31: 2021. The characterisation of isolates as multiple drug resistance (MDR) was done according to well-established criteria as described in the online Supplementary file.

Statistical analysis

The biofilm expression intensities among the three groups were based on SCC categories of the *S. uberis* isolates (*n* = 169), applying the Pearson $\chi 2$ test. The MIC data analysis used the LabPro software of the MicroScan 40 WalkAway system (Beckman Coulter, California, USA) to determine the MIC 90 values. The MIC 50 was calculated manually, using the automated MIC system results (MicroScan 40 WalkAway system, Beckman Coulter, USA).

Results and discussion

Biofilm expression and intensity

All of the *S. uberis* isolates that were assessed expressed biofilm, although with varying intensities per SCC group under in vitro conditions (Table 1). Biofilm expression for the *S. uberis* isolates was weak in 80 (47.3%), moderate in 59 (34.9%) and strong in 30 (17.8%) of isolates. Moore (2009) reported 100% biofilm expression by *S. uberis* isolates obtained in USA and Germany. During 2019 UBAC (HIPRA), a vaccine against *S. uberis* mastitis, was registered in South Africa with biofilm adhesion components, including lipoteichoic acid. This vaccine may be a positive method in South Africa for combating *S. uberis* IMI as all *S. uberis* isolates tested positive for biofilm expression.

Biofilm and somatic cell count

No significant differences ($P > 0.05$) were established between the proportions of biofilm intensity among the three SCC groups of isolates, suggesting that biofilm expression intensities are independent of the SCC category level of the isolates tested (Table 1). These findings partially agree with those of a similar study from Argentina by Fessia et al. (2020), comparing biofilm expression of *S. uberis* isolated from clinical and subclinical mastitis cases. Those authors established that 71.4 and 84.6% of *S. uberis* isolates produced weak biofilm, 9.6 and 7.7% moderate to strong, whereas 19.0 and 7.7% were non-producers for clinical and subclinical (> 250 000 cells/ml) mastitis isolates, respectively.

The current study established that none of the *S. uberis* tested were non-biofilm producers, whereas the percentage of moderate-to-strong biofilm producers was considerably higher than in the Argentinian study, varying between 51.0 and 55.2% for the three SCC groups. Neither study could establish a significant relationship between biofilm formation and the SCC levels. A reason for various levels of biofilm expression may be using various broths and incubation periods in the two studies. Fessia et al. (2020) used Todd-Hewitt broth with 1% yeast extract and a 24 h incubation period compared to the tryptose soy, with 10% glycerol and a 48 hr incubation in the current study.

Antimicrobial susceptibility testing

Most (91.3%) *S. uberis* isolates in this study demonstrated high susceptibility rates to the 23 antimicrobials tested. The susceptibility to penicillin was slightly lower at 151 (87.8%), than other products (Table 2). A Taiwanese study by Hsieh et al. (2019) determined *S. uberis* isolates to be 80.9% susceptible to penicillin. This was despite penicillin being used for decades in dairy cattle in Taiwan, both as intramammary and parenteral therapy, as well as

Table 1. Biofilm Expression and Intensity of *Streptococcus uberis* ($n = 169$) Isolates

S. uberis isolates	Sample size *n*	Weak biofilm expression *n* (%)	Moderate biofilm expression *n* (%)	Strong biofilm expression *n* (%)
Group A				
Isolates from clinical Mastitis	58	26 (44.8)	23 (39.7)	9 (15.5)
Group B				
Isolates from subclinical mastitis (SCC ≥ 300 000 cells/ml)	60	29 (48.3)	16 (26.7)	15 (25.0)
Group C				
Isolates from IMI(SCC < 300 000 cells/ml)	51	25 (49.0)	20 (39.2)	6 (11.8)
Total *n* (%)	169	80 (47.3)	59 (34.9)	30 (17.8)

IMI, intramammary infections.

its extensive use in human medicine (Hsieh et al., 2019). Minst et al. (2012) reported 100% susceptibility to penicillin and ampicillin in Germany, where β-lactams are the first line of defence for most Gram-positive infections. Variations in antibiotic resistance could be due to various regional locations, time of the study, and the pathogen management level on the farm.

It was found that 36/172 (20.93%) of isolates indicated resistance, however, 25/172 (14.5%) of these isolates were resistant to one or two antibiotics only. The 6.4% *S. uberis* isolates with MDR were resistant to between three and 12 antimicrobial groups and caused 75% of the resistant test results (Table 2). Bacterial resistance to various antibiotic groups or classes can occur inherently owing to the absence of binding sites or other pharmacological characteristics. Acquired resistance posing a risk of transmission to the human population is a major public health concern (Dhingra et al., 2020).

MDR was highest in these antimicrobial classes: tetracyclines (26), cephalosporins (25) and lincosamides (24). It was less in the ß-lactams (17), antimycobacterials (15) and macrolides (13) (Table 2 & online Supplementary Table S1). Cephalosporins, ß-lactams and tetracyclines were present in intramammary products available and registered for use in South Africa at the time of the study, whereas lincosamides, antimycobacterials and macrolides were unobtainable. These three antimicrobial groups are mainly used in human medicine, so this finding suggests possible bacterial transfer between humans and animals. Lincosamides are used in human medicine for infections caused primarily by streptococci and staphylococci. A South African human study by Bolukaoto et al. (2015) indicated a high resistance to erythromycin and clindamycin. Antimycobacterials treat mycobacterium infections in humans whereas macrolides are used as first-line treatment of atypical community-acquired pneumonia and acute non-specific urethritis (described in more detail in the online Supplementary File).

S. uberis was more resistant to chloramphenicol at MIC 90 and MIC 50 and least resistant to clarithromycin and daptomycin at MIC 90 and MIC 50 (online Supplementary file Table S1). *S. uberis* is a known pathogen causing mastitis in dairy cattle and is rarely associated with human infections. A plausible explanation is that erythromycin and clindamycin harbour constitutive macrolide, lincosamide and streptogramin B (cMLSB) and inducible macrolide, lincosamide and streptogramin B (iMLSB) in their phenotypes, important for resistance (Bolukaoto et al., 2015). Therefore, a one health approach is required among human and veterinary professionals in AMR surveillance strategy (Perovic and Schultsz, 2016).

The positive outcome of this study suggests that farmers can be encouraged to keep practising prudent use of intramammary remedies and other products in the dairy industry.

Biofilm and antimicrobial resistance

Numerically, the moderate biofilm producers exhibited higher sensitivity to antimicrobials than either the weak or strong biofilm producers. No meaningful statistical results could be obtained owing to low sample numbers so this rather surprising finding may be a chance observation (Table 2). Studies on human-origin bacteria (described in the online Supplementary File) concluded that the mechanism of biofilm-associated AMR is multifactorial and may vary from organism to organism. The practical implications of biofilm formation are

Table 2. Summary of Biofilm Expression and Antibiotic Resistance Patterns of *Streptococcus uberis* Isolates

Antibiotics (Product)	Biofilm Weak (80)			Biofilm moderate (59)			Biofilm strong (30)		
	S	*I*	*R*	*S*	*I*	*R*	*S*	*I*	*R*
	N (%)	*N* (%)	*N* (%)	*N* (%)	*N* (%)	*N* (%)	*N* (%)	*N* (%)	*N* (%)
Amoxicillin/Clav[1]	77 (96.25)	0 (0.00)	3 (3.75)	59 (100)	0 (0.00)	0 (0.00)	29 (96.67)	0 (0.00)	1 (3.33)
Ampicillin[1]	73 (91.25)	2 (2.50)	5 (6.25)	59 (100)	0 (0.00)	0 (0.00)	28 (93.33)	1 (3.33)	1 (3.33)
Azithromycin[4]	75 (93.75)	1 (1.25)	4 (5.00)	59 (100)	0 (0.00)	0 (0.00)	28 (93.33)	0 (0.00)	2 (6.67)
Cefepime[2]	76 (95.00)	0 (0.00)	4 (5.00)	59 (100)	0 (0.00)	0 (0.00)	27 (90.00)	1 (3.33)	2 (6.67)
Cefotaxime[2]	75 (93.75)	1 (1.25)	4 (5.00)	59 (100)	0 (0.00)	0 (0.00)	27 (90.00)	1 (3.33)	2 (6.67)
Ceftriaxone[2]	74 (92.50)	3 (3.75)	3 (3.75)	59 (100)	0 (0.00)	0 (0.00)	28 (93.33)	0 (0.00)	2 (6.67)
Cefuroxime[2]	75 (93.75)	0 (0.00)	5 (6.25)	59 (100)	0 (0.00)	0 (0.00)	27 (90.00)	0 (0.00)	3 (10.00)
Chloramphenicol[9]	77 (96.25)	0 (0.00)	3 (3.75)	59 (100)	0 (0.00)	0 (0.00)	28 (93.33)	0 (0.00)	2 (6.67)
Clarithromycin[4]	75 (93.75)	1 (1.25)	4 (5.00)	59 (100)	0 (0.00)	0 (0.00)	28 (93.33)	0 (0.00)	2 (6.67)
Clindamycin[5]	76 (95.00)	0 (0.00)	4 (5.00)	55 (93.20)	0 (0.00)	4 (6.78)	28 (93.33)	0 (0.00)	2 (6.67)
Daptomycin[10]	74 (92.50)	0 (0.00)	6 (7.50)	57 (96.60)	0 (0.00)	2 (3.39)	28 (93.33)	0 (0.00)	2 (6.67)
Erythromycin[4]	73 (91.25)	1 (1.25)	6 (7.50)	59 (100)	0 (0.00)	0 (0.00)	28 (93.33)	0 (0.00)	2 (6.67)
Levofloxacin[6]	78 (97.50)	0 (0.00)	2 (2.50)	59 (100)	0 (0.00)	0 (0.00)	29 (96.67)	0 (0.00)	1 (3.33)
Linezolid[12]	77 (96.25)	0 (0.00)	3 (3.75)	59 (100)	0 (0.00)	0 (0.00)	28 (93.33)	0 (0.00)	2 (6.67)
Meropenem[7]	78 (97.50)	0 (0.00)	2 (2.50)	59 (100)	0 (0.00)	0 (0.00)	28 (93.33)	0 (0.00)	2 (6.67)
Minocycline[3]	78 (97.50)	0 (0.00)	2 (2.50)	59 (100)	0 (0.00)	0 (0.00)	29 (96.67)	0 (0.00)	1 (3.33)
Moxifloxacin[6]	78 (96.25)	0 (0.00)	2 (3.75)	59 (100)	0 (0.00)	0 (0.00)	29 (96.67)	0 (0.00)	1 (3.33)
Penicillin[1]	70 (87.50)	4 (5.00)	6 (7.50)	55 (93.2)	4 (6.78)	0 (0.00)	24 (80.00)	5 (16.67)	1 (3.33)
Pristinamycin[11]	75 (93.75)	2 (2.50)	3 (3.75)	57 (96.6)	2 (3.39)	0 (0.00)	27 (90.00)	1 (3.33)	2 (6.67)
Rifampin[13]	76 (95.00)	0 (0.00)	4 (5.00)	57 (96.6)	2 (3.39)	0 (0.00)	24 (80.00)	0 (0.00)	6 (20.00)
Tetracycline[3]	74 (92.50)	1 (1.25)	5 (6.25)	55 (93.2)	0 (0.00)	4 (6.78)	29 (96.67)	0 (0.00)	1 (3.33)
Trimeth/Sulp[8]	74 (92.50)	4 (5.00)	2 (2.50)	53 (89.8)	0 (0.00)	6 (10.17)	27 (90.00)	2 (6.67)	1 (3.33)
Vancomycin[10]	76 (95.00)	0 (0.00)	4 (5.00)	55 (93.2)	3 (5.08)	1 (1.70)	29 (96.67)	0 (0.00)	1 (3.33)

S, Sensitive; I, Intermediate; R, Resistant; Antimicrobial groups: Penicillins = 1; Cephalosporins = 2; Tetracycline = 3; Macrolides = 4; Lincosamides = 5; Fluoroquinolons = 6; Carbapenems = 7; Sulphonamides = 8; Chloramphenicols = 9; Polypeptide = 10; Streptogramin = 11; Oxazolidinones = 12; Antimycobacterials = 13.

that alternative control strategies must be devised for testing the organism's susceptibility within the biofilm, in addition to devising treatments that alter its structure, as envisaged by vaccines targeting biofilm.

In conclusion, the main outcome of this study was the 100% biofilm expression of *S. uberis*. No significant relationship was established between *S. uberis* isolates from the three SCC categories of clinical and subclinical intramammary infection. Farmers may confidently use the new vaccine, targeting the biofilm structure. High antimicrobial susceptibility of 91.3% was present in the 23 antimicrobials tested. Most resistance (75%) was established in 6.4% of isolates. These isolates were resistant to between three and 12 antimicrobial groups. MDR was mainly against tetracyclines, cephalosporins and ß-lactams used in bovine intramammary treatment and lincosamides, anti-mycobacterials and macrolides mainly used in human medicine. Resistance to antibiotics used only in humans emphasises the importance of applying the one health approach. The low positive resistance can motivate veterinarians and dairy farmers to continue subscribing to the prudent use of antimicrobials.

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