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

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SUPPLEMENTARY FILE

Detailed Methodological descriptions

Data source

Streptococcus uberis isolates were initially identified by classical microbiology phenotypic methods (International Dairy Federation, 1985) and confirmed by the MALDI-TOF MS (Bruker Daltronics, Bremen, Germany) (Department of Plant and Soil Sciences, University of Pretoria, South Africa) (van Dyk, 2015).

Detection of biofilm formation

The *S. uberis* isolates were revived from glycerol stock cultures kept at -80^{0}C, sub cultured onto blood tryptose agar (BTA) agar plates and incubated at 37 ^{0}C for 24 hrs. Following incubation, single colonies were transferred from the plates and inoculated into 15 mL tubes containing 10 mL trypticase soy broth (TSB) and 10% glycerol (Kwon *et al.* 2017). Two American Type Culture Collection (ATCC) of known biofilm former strains namely *Staphylococcus epidermidis* (ATCC 35984) and *S. uberis* (ATCC 700407) were included as positive controls while TSB without isolates served as a negative control (Olawuwo *et al.* 2022). All samples were tested in triplicates with two replicates (n = 6).

The working stock was prepared by diluting the culture with TSB plus 10% glycerol (Kwon *et al.* 2017) to a final absorbance of 0.02 (approximately 10^6 CFU/mL) @ 590 nm using a microplate reader (BioTek Synergy, USA).

The wells of the flat bottom plates were washed three times using sterile distilled water while gently flicking the plates after each wash and left to dry for about 15 min. Plates were then placed into an oven drier, set at 60^0C for 45 minutes (Olawuwo *et al.* 2022).

A volume of 100 µL crystal violet (0.2 %) solution was added to each well for 15 minutes to fix the cells (Stepanović *et al.* 2007). The stain was gently rinsed off using distilled sterile water and left to dry for 15 min at room temperature (Stepanović *et al.* 2007). Then 125 µL of 96% ethanol was added to each well to elute the stain and left covered for 15 minutes (Stepanović *et al.* 2007). The absorbance of the plates was determined at a wavelength of 590 nm using a microplate reader (Sandasi *et al.* 2010).

Biofilm interpretation

The two reference ATCC strains were used as positive controls (Phophi *et al.* 2019).

ODc= Mean (Neg Control) + 3 x (Std Dev Neg Control). A final ODc = 0, 0734 was considered the cut-off (Stepanović *et al.* 2007).

Biofilm production was categorised as follows:

– Negative for biofilm production: OD ≤ ODc, that is all strains with OD values below 0.0734

– Weak biofilm production: ODc: < OD ≤ 2 × ODc, that were all strains with OD values above 0.0734 to 0.1468

– Moderate biofilm production: 2 × ODc < OD ≤ 4 × ODc that were all strains with OD values above 0.468 to 0.2936

– Strong biofilm production: OD > 4 × ODc that is all strains with OD values above 0.2936 (Stepanović *et al.* 2007).

Antimicrobial susceptibility testing

The *S. uberis* isolates were subjected to antimicrobial susceptibility to a commercially available panel of 23 antibiotics in (µg/mL) as per package insert (Micro STREP plus Panel Type 6, Beckman Coulter). The selected panel contained most of the antibiotics available as intramammary products in South Africa. An additional two reference strains of *S. uberis* ATCC 27958 and ATCC 700407 (Thermo Fischer Scientific, Massachusetts, United States) were used as controls. The susceptible breakpoints used for the MIC for each antibiotic are as stipulated in (CLSI VET01S ED5:2020; CLSI M100: ED 31:2021).

The MIC 50 represents the MIC value at which ≥*50% of* the isolates in a test population *are* inhibited, and it is equivalent to the median MIC value. The MIC 90 represents the MIC value at which ≥90% of the isolates in the test population are inhibited (Schmidt 1987). The MIC breakpoints (chosen concentration [mg/L] of an antibiotic which defines whether a species of bacteria is susceptible or resistant to the antibiotic.

Statistical analysis

The statistical software GenStat® (VSN International, 2019) was used for the Pearson chi square test analyses. The Generalized linear models (GLM) was applied to the sensitivity proportions using the binomial distribution and the logit link function

and predicted means were compared using Fisher's protected least significant difference test at the 5% level ($p < 0.05$) of significance (Freund, Mohr & Wilson, 2010). However, the sample numbers per category were too few for any meaningful results of the GLM analysis.

Supporting Results

Biofilm expression and intensity

Moore (2009) in a USA study and Schönborn *et al*. (2017) in Germany both also reported 100% biofilm expression by *S. uberis* isolates.

Antimicrobial susceptibility testing

A study by Minst *et al*. (2012) reported 100% susceptibility to penicillin and ampicillin in a German study where ß -lactams are considered the first line of defence for most Gram-positive infections. Variations in antibiotic resistance could be due to various regional locations, time of study and level of management of the various pathogens on farm (Karzis *et al*., 2019).

Antimycrobacterials are used to treat mycobacterium infections in humans (Assefa, 2022) while macrolides are used as first-line treatment of atypical community acquired pneumonia and acute non-specific urethritis (Ismail *et al.* 2018).

Biofilm expression and intensity

All *S. uberis* isolates tested, expressed biofilm under *in vitro* conditions with varying degrees of intensities (weak, moderate and strong) per group (Table 1).

A majority of all the *S. uberis* isolates showed a high susceptibility to the panel of antimicrobial products used (Table 2). A total of 36/172 (20.93%) isolates showed

some resistance, however, 24/172 (13.95%) of these resistant isolates were only resistant to 1 or 2 antibiotics. There were 11/172 (6.4%) *S. uberis* isolates which showed multi drug resistance (resistance to 3 or more antibiotic classes) (Table 2).

Table S1. Distribution of minimum inhibitory concentrations (MIC) cumulative percentage inhibited by antibiotic level for *S. uberis* (n=172)

Antibiotic/ Product	Resistance % (n)	Intermediate % (n)	Susceptible % (n)	Distribution MIC (µg/mL) (n=172)																			MIC 90%	MIC 50%
				0.03	0.06	0.12	0.25	0.5	1	2	4	6	8	16	25	32	64	100	128	256	500	1000	2000		
Amoxycillin/ Clavulanic acid	2.3 (4)	0.0	97.7 (168)	-	-	-	-	-	98	98	98		-										1	1
Ampicillin	4.1 (7)	2.3 (4)	93.6 (161)	-	40	85	94	95	97	97	97		-										0.25	0.12
Azithromycin	3.5 (6)	0.0	96.5 (166)	-	-	91	95	96	97	98	-		-										0.12	0.12
Cefepime	3.5 (6)	1.2 (2)	95.3 (164)	-	-	-	80	96	96	97	-		-										0.5	0.25
Cefotaxime	3.5 (6)	1.2 (2)	95.3 (164)	-	-	-	45	86	95	97	-		-										1	0.5
Ceftriaxone	2.9 (5)	2.3 (4)	94.8 (163)	-	-	-	49	80	95	97	-		-										1	0.5
Cefuroxime	4.7 (8)	0.0	95.3 (164)	-	-	-	48	89	95	96													1	0.5
Chloramphenicol	2.9 (5)	0.0	97.1 (167)	-	-					89	97	-	97										4	2
Clarithromycin	3.5 (6)	0.6 (1)	95.9 (165)	-	-	95	96	97	97	98	-												0.12	0.12
Clindamycin	5.8 (10)	0.0 (0)	94.2 (162)	-	92	94	94	94															0.06	0.06
Daptomycin	5.8 (10)	0.0	94.2 (162)	-	-	-	91	94	94	94	-		-										0.25	0.25
Erythromycin	4.1 (7)	1.1 (2)	94.8 (163)	-	95	95	95	96	-	-	-		-										0.06	0.06
Levofloxacin	1.7 (3)	0.0	98.3 (169)	-	-	-	-	85	98	98	98		-										1	0.5
Linezolid	2.3 (4)	0.0	97.7 (168)	-	-	-	95	97	98	98	98		-										0.25	0.25
Meropenem	2.3 (4)	0.0	97.7 (168)	-	-	-	95	97	98	98	-		-										0.25	0.25
Minocycline	8.1 (14)	0.0	91.9 (158)	-	-	-	-	90	92	94	-		-										0.5	0.5
Moxifloxacin	1.7 (3)	0.0	98.3 (169)	-	-	-	95	98	98	98	-		-										0.25	0.25
Penicillin	4.1 (7)	8.1 (14)	87.8 (151)	25	62	88	93	95	95	96	97		-										0.25	0.06
Pristinamycin	2.9 (5)	2.9 (5)	94.2 (162)		-	-	-	-	94	97	-		-										1	1
Rifampin	8.7 (15)	0.0	91.3 (157)		-	-	-	90	91	92	-		-										0.5	0.5
Tetracycline	7.0 (12)	0.6 (1)	92.4 (159)		-	-	-	-	90	92	93		-										1	1
Trimethoprim / Sulphamethoxazole	0.0 (0)	7.6 (13)	92.4 (159)		-	-	92	95	95	97	-		-										0.25	0.25
Vancomycin	2.9 (5)	0.0	97.1 (167)		-	-	17	90	97	97	97		-										0.5	0.5

Moderate biofilm producers had numerically observed higher sensitivity than weak and strong biofilm producers although meaningful statistical results could not be obtained due to low sample numbers in certain categories.

The highest percentage of resistant *S. uberis* isolates was found to rifampin (8.7%), minocycline (8.1%) and tetracycline (7.0%). On the other hand, all the isolates tested were susceptible to trimethoprim/sulfamethoxazole (0%). Most of the tested isolates were susceptible to levofloxacin and moxifloxacin (98.3%), amoxicillin/clavulanic acid, linezolid, meropenem (97.7%). Only a few (11 isolates, 6.4%), were multidrug resistant (≥ 3 groups of antimicrobials). The most frequent combination of resistances according to classes; Tetracyclines (26), Cephalosporins (25) and Lincosamides (24), medium resistant classes; ß-lactams (17), Antimycobacterial (15) and Macrolides (13) and least resistance classes; Fluoroquinolones (6), (Phenols; Streptogramin; Glycopeptides (5)) and Oxazolidinones (4), Folate path inhibitors (4) and Carbapenems (3).

Supporting literature

Introduction

Bovine mastitis can be caused by a diverse group of pathogens. *Streptococcus uberis* (*S. uberis*) is one of the predominant pathogens associated with both subclinical and clinical mastitis (Ruegg, 2011).

Although *S. uberis* is considered to be an environmental pathogen, its host adapted strains have the ability to adhere to the epithelial cells of the mammary gland causing persistent and recurrent infections (Jamal *et al*. 2018).

This bacteria can colonise multiple body sites including the intestinal and genital tracts and the mammary gland (Ward *et al.* 2009). The ability of *S. uberis* to survive in the environment is favoured by the presence of a hyaluronic acid capsule, an extracellular virulence factor (Calvinho *et al.* 1998). *S. uberis* is excreted in bovine faeces and can be present in both bedding material and on dairy pastures (Lopez-Benavides *et al.* 2007).

Antimicrobial resistance in streptococci

Reports in New Zealand (McDougall et al., 2020) and Switzerland (Haenni et al., 2018) are revealing reduced sensitivity or resistance to both classes of antibiotics. Resistance to penicillin, amoxicillin / clavulanic acid, ampicillin, erythromycin, and clindamycin has been shown in streptococci for mastitis studies done in Egypt (Saed & Ibrahim, 2020) but not in Uruguay (Gianneechini et al., 2002).

Biofilm and Antimicrobial resistance

Other virulence factors of *S. uberis* such as activation genes can transfer antibiotic resistance genes within members of the biofilm micro-community (Lebeaux *et al.* 2014).

Both, early onset of treatment and a prolonged treatment period can be expected to improve cure rates (Melchior et al., 2006).

Studies on bacteria from human origin have concluded that the mechanism of biofilm-associated antimicrobial resistance seems to be multifactorial and may vary from organism to organism (Patel, 2005). The practical implications of biofilm formation are that alternative control strategies must be devised for testing the

susceptibility of the organism within the biofilm and treating the biofilm to alter its structure (Donlan, 2000), as is done by vaccines targeting biofilm.

The characterisation of isolates as multi resistant also known as MDR, was done according to the well-established criteria (Magiorakos *et al.* 2012).

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