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Efficacy of Resveratrol and Ursolic Acid on Biofilm Inhibition and Antimicrobial Resistance of *Streptococcus uberis*

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ABSTRACT

Background: Bovine mastitis is a multifactorial and is one of the most challenging disease. It can be caused by many different bacterial species, the most common of which are *Staphylococcus* species and *Streptococcus* species. The prevalence of different species varies temporally, geographically and also due to control measures adopted in herds. The present study is on the biofilm forming *Streptococcus uberis* causing mastitis and the effect of antibiofilm agents on the antimicrobial resistance of the microorganisms.

Methods: The isolates were identified by Polymerase chain reaction (PCR). Two antibiofilm agents, resveratrol and ursolic acid (UA) each at two concentrations (30 µg/ml, 100 µg/ml) were used for the study. Biofilm formation and rate of biofilm inhibition was detected using quantitative microtiter plate (MTP) assay and biofilm gene (*lux S)* was detected using PCR. The isolates treated with antibiofilm agents were subjected to standard disc diffusion test with 7 antibiotics and the change in antibiotic resistance was studied.

Result: The mean±SE values of inhibition rates of 29 *S. uberis* isolates by 30 µg/ml UA, 100 µg/ml UA, 30 µg/ml resveratrol, 100 µg/ml resveratrol were 33.96±3.17%, 57.40±2.8%, 31.35±3.12% and 46.28±3.47%, respectively. Biofilm inhibiting agents along with all antibiotics had reduced antimicrobial resistance by 1.5-2 times on *in vitro* antibiotic resistance testing by disc diffusion at 100 µg/ml concentration. The antibiofilm agents were found to be very effective to control antibiotic resistance of *S. uberis* from mastitic milk samples *in vitro*.

Key words: Antibiotic resistance, Biofilm detection, Biofilm inhibition, *luxS* gene, Resveratrol, *Streptococcus uberis*, Ursolic acid.

INTRODUCTION

Mastitis is an important disease affecting diary animals causing a huge economical loss. Mastitis also has got huge public health significance rather than its economic importance (Sudhan *et al.,* 2005). There are many bacterial, viral and other infectious agents causing mastitis. The most common etiological agent is *Staphylococcus aureus* followed by Streptococcus species causing about 15% of mastitis in different parts of India. *S. uberis*, a primary environmental pathogen is a major cause of mastitis in Dairy cattle. Chronic subclinical mastitis caused by *S. uberis* was extremely costly and difficult to treat (Steeneveld *et al.,* 2007).

Intramammary infections caused by *S. uberis* can vary from subclinical to clinical mastitis and it is about 11.86% in Finland (Koivula *et al.,* 2007). This is also found to be most common cause of clinical mastitis in the UK (23.5%) (Bradley *et al*., 2007). *S. uberis* is found to be more often present in chronic infections than in new subclinical cases (Persson *et al*., 2011).

Biofilm formation has a key role in antibiotic resistance of the microbes. *S. uberis* forms biofilms *in vitro* on abiotic surface and the substrates did not appear to have an effect on its ability to form biofilms under the conditions (tryptic soy broth, 5% sucrose, 5% lactose) and assays used. Also, *S. uberis* has the ability of to attach to host cell surface through the *S. uberis* adhesion molecule (SUAM) (Moore, 2009). Traditional antibiotic therapy acts by destructing planktonic cells only, leaving the attached forms to propagate within the biofilm and continue to disseminate when treatment is stopped (Wang *et al.,* 2009). There is evidence that quorum sensing is important for the construction and/or dissolution of biofilm communities in some species. So that quorum sensing inhibitors have the potential ability to be used as an adjuvant in antimicrobial therapy (Parsek and Greenberg, 2005).

Resveratrol (3,5,4′ -trihydroxystilbene) is a stilbene naturally present in foodstuffs with described antimicrobial activity against several pathogens (Ferreira *et al.,* 2014; Paulo *et al*., 2010). Resveratrol has got important pharmacology effects, such as anticancer, antioxidation, immunoregulation, anti-inflammation and antibacterial activity (Horiuchi *et al.,* 2007). The ability of resveratrol and its inclusion complex in the inhibition of biofilm formation and in the dispersion of established biofilms as well as its

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quorum sensing inhibition activity was evaluated. The inclusion complex and resveratrol display a bacteriostatic or bactericidal effect, with this effect being time and concentration dependent and with a faster killing kinetics in the case of resveratrol in comparison to the inclusion complex (Duarte *et al.,* 2015). Ursolic acid (UA) is a relatively nontoxic active ingredient of many medicinal plants and has a broad range of pharmacological effects, including protection against liver injury, antitumor activity, inhibition of mutagenesis in bacteria, anti-inflammation and antiulcer activity (Ren *et al.,* 2005). Zhou *et al.* (2013) reported the enhancing antimicrobial and anti-biofilm efficacy of UA and xylitol, against *S. mutans* and *S. sobrinus* in the oral environment by synergistic inhibition. The present study is on the effect of antibiofilm agents *viz*., UA and resveratrol in combination with 7 antibiotics on antibiotic resistance of biofilm forming *S. uberis* isolated from mastitic milk samples of cows and buffalos.

MATERIALS AND METHODS

Sample collection

Milk samples were collected with owner's consent as per standard milk collection procedure. Samples were collected aseptically from bovine mastitic cases presented to veterinary hospitals and from farms in and around Krishna, Guntur and west Godavari districts, Andhra Pradesh. The study was conducted in the Department of Veterinary Microbiology, NTR College of Veterinary Sciences, Gannavaram, Andhra Pradesh.

Bacterial isolation and identification

Approximately 10 ml of milk was collected aseptically from clinical cases into sterile vials. Collected samples from each quarter were transported on ice and immediately cultured or stored at 4°C until cultured/enriched. Milk samples were centrifuged at 2000 g for 10 minutes at 37°C, supernatant was discarded and 5 ml of brain heart infusion (BHI) broth was added to the sediment and incubated at 37°C for 24 hr (Cruickshank *et al.,* 1975). Selective isolation was done by inoculating 0.9 ml of *Streptococcus* selective (SS) broth with 0.1 ml of culture from the BHI broth and incubated at 37°C in an anaerobic jar for 24 hr. The morphology of the organisms was studied by Gram's staining. SS broth with Gram positive cocci in chain were further inoculated on to Edward's medium. The *S. uberis* isolates were identified phenotypically and genotypically. The cultures showing greyish, pinpointed colonies and/or aesculin hydrolysis on Edward's medium were tentatively identified as *Streptococcus* species. The suspected isolates of *Streptococcus* species were further identified by various biochemical tests *viz.,* catalase test, ninhydrin test, sodium hippurate hydrolysis test and type of haemolysis on 7% sheep blood agar.

DNA extraction

DNA was extracted by High salt method (Anand Kumar, 2009) and re-suspended in 40 µl sterile distilled water and stored at -20°C till use. The procedure followed was,

1. Two ml of enriched bacterial culture in tryptic soya broth (TSB) was first washed (at 5,000 rpm for 10 minutes) with Phosphate Buffered Saline in fresh Eppendorf tube (2.0 ml) and then washed with TKM-1 solution (Appendix) at 5,000 rpm for 10 minutes.
2. The bacterial cell pellet was suspended in 100 µl of TKM-2 solution (Appendix) and incubated for 15 min at 37°C.
3. Then, 50 µl of 10% Sodium Dodecyl Sulphate was added and mixed well. Subsequently, 250 µl of 6M Sodium Chloride was added, mixed well and centrifuged at 10,000 rpm for 5 min.
4. The supernatant containing nucleic acid was transferred to fresh microcentrifuge tube (1.5 ml) then 2 volumes of 100% ethanol were added, mixed thoroughly and centrifuged at 10,000 rpm for 5 min at 4°C.
5. The DNA pellet thus obtained was washed twice with 75% ethanol (at 5,000 rpm for 10 minutes) and finally re-suspended in 40 µl sterile distilled water and stored at -20°C till use.

Measurement of DNA concentration and purity

The concentrations of DNA were measured with Nanodrop 20°C (Thermo Scientific, USA) and adjusted to 50 ng/µl for further molecular studies. Pure DNA samples (with an optical density ratio of 1.8 to 2 at 260/280 nm) were stored at -20°C, until further use.

Identification of isolates by PCR

The nucleotide sequence of primers used for detection of *S. uberis* (Sub 302/Sub 396) were, F- CGA AGT GGG ACA TAA AGT TA, R- CTG CTA GGG CTA AAG TCA AT (Riffon *et al.,* 2001) coding for 23S rRNA, with specific annealing temperatures of 53°C. The isolates with *luxS* gene responsible for biofilm formation in were identified by the specific primer with sequence F- TTT GAT GTT CGC TTG GTT CA, R- AGT TTT GCC CAT TCT TTT GC (Moore, 2009). The time temperature combinations used for PCR are given in Table 1. The PCR amplicons were analysed by electrophoresis on 1.7% agarose gel stained with 0.5 µg of ethidium bromide/1ml in Tris-Borate EDTA (TBE) buffer and then visualised by UV gel documentation system (Bio-Rad).

Biofilm detection in *S. uberis* isolates

Biofilm forming isolates were identified by MTP assay (Christensen *et al.,* 1985; Merrit *et al.*, 2005) with minor modifications by Moore (2009). Quantification of biofilm/nonbiofilm producing colonies was done according to Milanov *et al.* (2015). Cut-off OD is defined as three standard deviations above the mean OD of the negative control. Isolates were classified as follows:

Non-biofilm producers (OD $\leq$ ODc)
Weak biofilm producers (ODc < OD $\leq 2 \times$ ODc)
Moderate biofilm producers ($2 \times$ ODc < OD $\leq 4 \times$ ODc)
Strong biofilm producers (OD > $4 \times$ ODc).

Biofilm inhibition in biofilm forming *S. uberis* isolates by microtiter plate assay

Biofilm forming isolates were identified by MTP assay. The antagonist effect of resveratrol and UA on biofilm formation and antibacterial resistance was studied by using different concentrations and by its comparison with the control group that did not receive any treatment. The rate of biofilm inhibition was studied by MTP assay. The *S. uberis* isolates were divided into five groups, group 1- control (isolates without receiving any treatment), group 2- Pure colonies with addition of 30 µg/ml UA, group 3- Pure colonies with addition of 100 µg/ml UA, group 4- Pure colonies with addition of resveratrol at concentration of 30 µg/ml, Group 5- Pure colonies with addition of resveratrol at concentration of 100 µg/ml.

Biofilm inhibition studies were conducted on the isolates by the following method, with minor modification from Moore (2009). Single colonies of each *S. uberis* isolate were inoculated into 5 ml of TSB, placed in a shaker incubator at 37°C and grown overnight to stationary phase. The following day bacterial cultures were diluted at 1:100 with sterile TSB broth with UA and resveratrol of which final concentrations were 100 µg/ml and 30 µg/ml. Hundred µl of the diluted culture were inoculated in a sterile 96-well U- bottom polystyrene plate (Tarsons, Kolkata) and incubated for 48 hours. Planktonic bacteria were removed by washing the plate four times with 100 µl of Phosphate buffered saline and any residual liquid was carefully aspirated. The plate was heat fixed for one hour at 60°C and stained with 100 µl Hucker's crystal violet solution for two minutes. The excess stain was removed by gentle shaking and washing with water until the water was clear. The plate was blotted dry and 100 µl of a solution containing 10% methanol and 7.5% acetic acid was added, the plate was shaken for one minute and placed in plate reader to record the absorbance at 563 nm. The inhibitory rates were calculated using the following formula:

$$\text{Inhibitory rate \%} = \frac{\text{OD563 nm of control - OD563 nm of sample}}{\text{OD563 nm of control}} \times 100\%$$

Antibiogram studies

The modified disc diffusion method of Kirby-Bauer was employed. Antibiotic susceptibility testing was performed using antibiotic test discs (Hi media, Mumbai) and interpretation was done according to 2007 CLSI guidelines. The disc diffusion test was done for each isolate on Mueller-Hinton agar. Initially antibiogram of isolates without treating with antibiofilm agents was done. For studying the variation in antimicrobial resistance when treated with antibiofilm agents, the *S. uberis* isolates were treated with 30 µg/ml of UA, 100 µg/ml of UA, 30 µg/ml of resveratrol and 100 µg/ml of resveratrol for 18 hours and antibiotic sensitivity test was done as per the protocol. The antibiotic impregnated discs and their concentrations used were Penicillin G (10 IU), Tetracycline (30 µg), Amoxycillin/Clavulanic acid (30/15 µg), Streptomycin (10 µg), Ceftriaxone (30 µg), Erythromycin (10 µg) and Enrofloxacin (10 µg).

RESULTS AND DISCUSSION

PCR detection of *S. uberis* and *luxS gene*

A total of 44 isolates were reacted to species specific *S. uberis* primers with product size of 94 bp (Fig 1). Out of those 29 isolates were biofilm formers and 15 were non biofilm formers. The study was focused on antimicrobial resistance of the biofilm forming isolates. Out of 29 *S. uberis* isolates tested for the presence of *luxS* gene 9 (31.03%) isolates reacted to *luxS* primers with product size of 317 bp (Fig 2). Satish Kumar (2016) also, reported the presence of

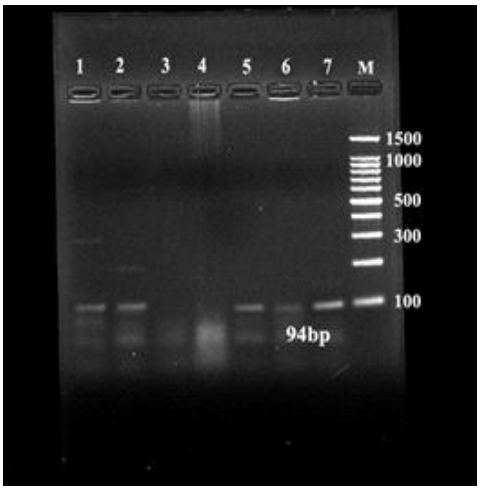

Fig 1: PCR amplification product of Sub 302 and Sub 396 oligonucleotide primers for *S. uberis*.
M= Marker; Lane 1= Sample 67; Lane 2= Sample 69; Lane 3= Negative; Lane 4= Negative control; Lane 5= Sample 27; Lane 6= Sample 72; Lane 7= Sample 76.

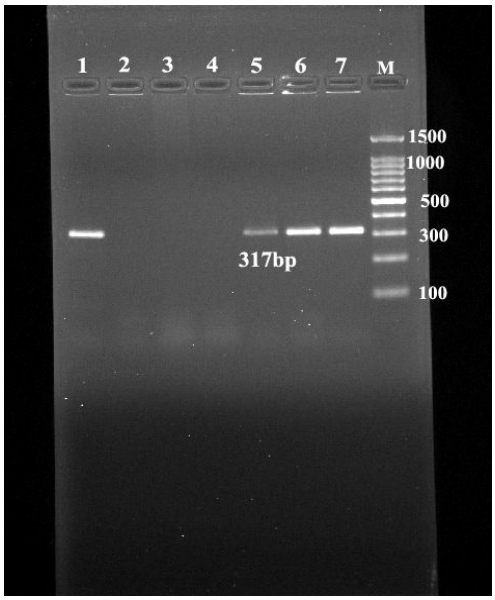

Fig 2: PCR amplification product of *luxS* gene of *S. uberis*.
M= Marker; Lane 1= Sample 41; Lane 2= Negative control; Lane 3= Negative; Lane 4= Negative; Lane 5= Sample 71; Lane 6= Sample 72; Lane 7= Sample 76.

luxS gene in 41.6% of *S. uberis* isolates in agreement with the present findings. In contrary Moore (2009) reported the presence of *luxS* gene in 96% of isolates.

Biofilm detection and inhibition studies

Of the selected 29 isolates on MTP assay 4 were strong biofilm producers, 2 were moderate biofilm producers, 23 were weak biofilm producers (Fig 3). The optical density of the isolates on MTP assay is represented graphically in Fig 4.

Mean±SE values of biofilm inhibition rates of UA at concentration of 30 µg/ml and 100 µg/ml was found to be 33.96±3.17% and 57.40±2.8% respectively. Mean±SE values of biofilm inhibition rates of resveratrol at concentration of 30 µg/ml and 100 µg/ml was found to be 31.35±3.12% and 46.28±3.47% respectively.

Antibiogram of the isolates

In vitro antibiotic sensitivity test of *S. uberis* revealed that 79.31% of the isolates were resistant to Ceftriaxone followed by Erythromycin (41.37%), Streptomycin (37.93%), Tetracycline (37.93%), Penicillin G (34.48%), Enrofloxacin (31.03%), Amoxycillin clavulanic acid (27.58%). The results were in agreement with Elango *et al.* (2010) but the resistance to Ceftriaxone was a contradiction from the study which showed only 7.34% resistance. In favour of the present study Jain *et al.* (2012) also reported high sensitivity of *Streptococcus* isolates to Gentamicin and Ampicillin. Increased antimicrobial resistance rates were identified in Streptococci against enrofloxacin, tetracycline, and erythromycin (Kabelitz *et al.*, 2021). Boireau *et al.* (2018) found the highest resistance level of *S. uberis* to enrofloxacin with 32.9%. Except for Tetracycline, resistance rates for the antibiotics tested were more or less stable over 10 years, there was a linear increase in resistance to Tetracycline between 2006 and 2016 from 15.7% to 20.4%.

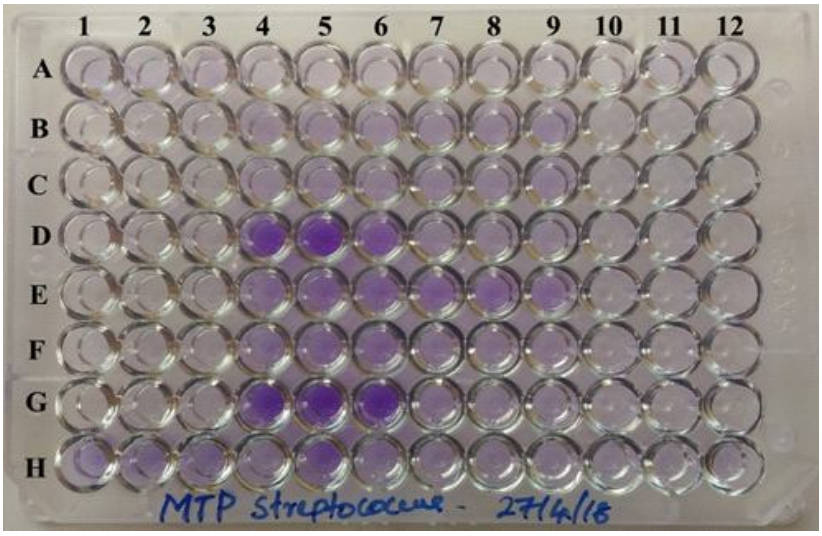

Fig 3: MTP of *S. uberis* isolates, G4 to G6- strong; E4 to E6- moderate; C4 to C6- weak; A1 to A3-non biofilm former and A10 to A12- negative control.

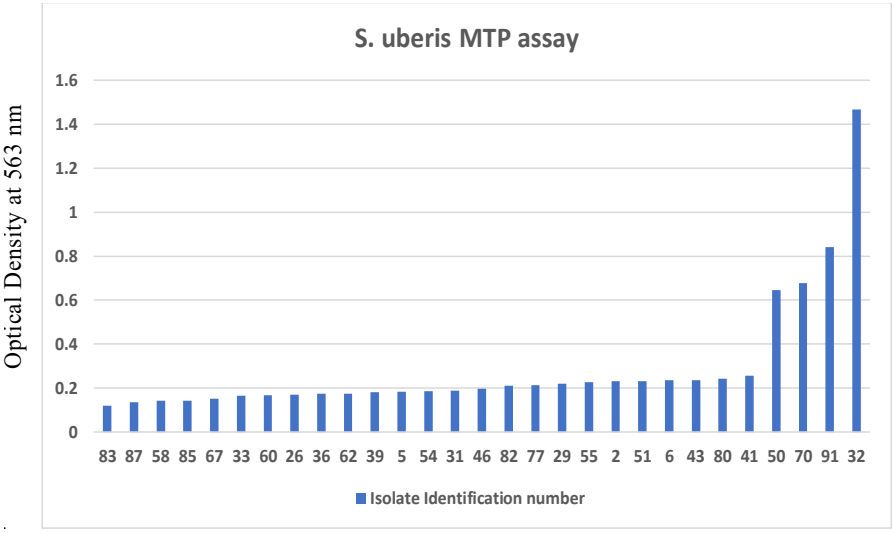

Fig 4: Graph showing Optical Density of *S. uberis* isolates on MTP assay.

The antibiotic resistance of the isolates to selected antibiotics has decreased drastically on treatment with antibiofilm agents. The reduction in antimicrobial resistance was observed highest with UA at 100 µg/ml concentration. The resistance to Ceftriaxone was reduced to 34.48% and to other antibiotics Erythromycin (27.58%), Streptomycin (13.79%), Tetracycline (20.68%), Penicillin G (17.24%), Enrofloxacin (27.58%), Amoxycillin clavulanic acid (17.24%). The variation in antimicrobial resistance pattern of *S. uberis* without treating with antibiofilm agents and after treatment with the specified concentrations are in Table 2 and 3 respectively. The variation in antimicrobial resistance of *S. uberis* is depicted graphically in Fig 5.

Mastitis is an important disease in diary animals causing huge economic loss mainly due to low production. Recurrent and subclinical infections increase the economic loss. Resistance of microorganism to antibiotics is the major concern of field veterinarians in the treatment of mastitis. Majority are trying a combination antibiotic therapy for good result. In terms of bovine mastitis, besides the invasion of

Table 1: PCR program.

Primer/gene	Product	Initial denaturation		Denaturation		Annealing		Extension	
		Temp	Time	Temp	Time	Temp	Time	Temp	Time
Sub (Sub 302 and Sub 396)	94 bp	94°C	2 min	94°C	30 sec	53°C	30 sec	72°C	30 sec
S. uberis (*luxS*)	317 bp	95°C	2 min	95°C	30 sec	58°C	1 min	72°C	1 min

PCR was run for 35 cycles and final extension step was maintained at 72°C for 10 min for all the oligonucleotide primer sets.

Table 2: Details of antimicrobial resistance pattern of *S. uberis* without treatment and after treating with UA in percentage.

Antibiotic disc	Group 1			Group 2			Group 3		
	S	I	R	S	I	R	S	I	R
P	13.79	51.72	34.48	17.24	58.62	24.13	41.37	41.37	17.24
AMC	72.41	0	27.58	79.31	0	20.68	82.75	0	17.24
CTR	17.24	3.44	79.31	24.13	10.34	65.51	51.72	13.79	34.48
TE	51.72	10.34	37.93	62.06	3.44	34.48	65.51	13.79	20.68
E	20.68	37.93	41.37	34.48	37.93	27.58	65.51	13.79	20.68
S	44.82	17.24	37.93	58.62	17.24	24.13	75.86	10.34	13.79
Ex	51.72	17.24	31.03	55.17	13.79	31.03	72.41	6.89	20.68

Table 3: Details of antimicrobial resistance pattern of *S. uberis* without treatment and after treating with resveratrol in percentage.

Antibiotic disc	Group 1			Group 4			Group 5		
	S	I	R	S	I	R	S	I	R
P	13.79	51.72	34.48	17.24	55.17	27.58	37.93	37.93	24.13
AMC	72.41	0	27.58	82.75	0	17.24	89.65	0	10.34
CTR	17.24	3.44	79.31	20.68	10.34	68.96	34.48	10.34	55.17
TE	51.72	10.34	37.93	58.62	10.34	31.03	65.51	3.44	31.03
E	20.68	37.93	41.37	34.48	27.58	37.93	51.72	17.24	31.03
S	44.82	17.24	37.93	51.72	20.68	27.58	68.96	3.44	27.58
Ex	51.72	17.24	31.03	65.51	13.79	20.68	75.86	6.89	17.24

Antibiotic disc: *P - Penicillin G, AMC - Amoxycillin clavulanic acid, CTR - Ceftriaxone, TE - Tetracycline, E - Erythromycin, S - Streptomycin, Ex - Enrofloxacin. S - Sensitive, I - Intermediate, R - Resistant, n - number of isolates.*

APPENDIX

TKM-1 solution

TRIS hydrochloride (Tris HCl) (pH- 7.6)	10 mM
Potassium chloride (KCl)	10 mM
Magnesium chloride ($MgCl_2$)	10 mM
Ethylene diamine tetra acetic acid (EDTA)	2 mM

TKM-2 solution

TRIS hydrochloride (Tris HCl) (pH- 7.6)	10 mM
Potassium chloride (KCl)	10 mM
Magnesium chloride ($MgCl_2$)	10 mM
Ethylene diamine tetra acetic acid (EDTA)	2 mM
Sodium chloride (NaCl)	0.4 mM

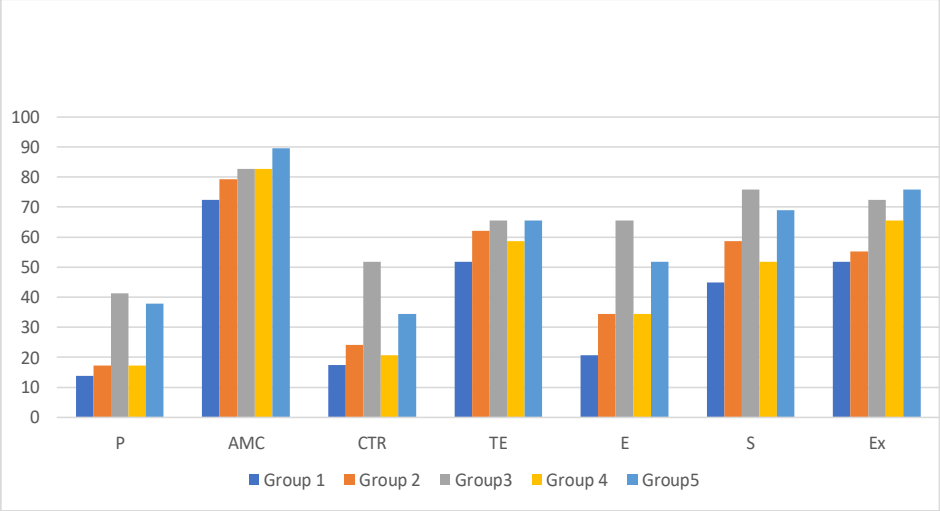

Fig 5: The graph showing the increase in the sensitivity to antibiotics using disc diffusion test.

mammary epithelial cells, survival of microorganisms in milk is an important step (Kabelitz *et al*., 2021). Recurrent infections are mainly due to biofilm formation by microorganism, which is not getting destroyed by the antibiotic therapy. Hygiene conditions, feeding and machine-milking are some of the factors associated with clinical cases caused by *S. uberis* (Barkema *et al*., 1999).

The most common phase of the production cycle in which dairy cows acquire an infection with *S. uberis* is dry period. Typically, *S. uberis* infections are manifested as acute mastitis, during the subsequent lactation (Hughes, 1999). Some infections may eventually turn cow-associated, due to its ability to persist within the mammary tissue. In other cases, the infection is short, but in any case, there is a high risk of re-infection. The risk for the dairy farm lies in the high rate of re-infection even though microbes remain susceptible to most antimicrobial agents (Kromker *et al*., 2014). Varhimo *et al.* (2011) described biofilm formation as one of the important virulence factors of some strains of *S. uberis*. Moreover, Crowley *et al.* (2011) demonstrated that the transition from planktonic to biofilm growth in the *S. uberis* 0140J strain correlated with an upregulation of several gene products that have been shown to be important for pathogenesis. Biofilm formation plays an important role in persistent and re-infections.

In this study, antibiogram of the isolates before and after treatment with the antibiofilm agents varied positively. The antibiotic resistance of the *S. uberis* isolates to selected antibiotics has decreased drastically on treatment. The increase in concentration of the antibiofilm agent again improved the sensitivity of the isolates to the antibiotics. It suggests that a combination of an antibiofilm agent along with the antibiotics may help to bring the case under control. Jyothi *et al.* (2018) studied the antagonistic effect of UA on *S. aureus* biofilms using MTP assay and found 71.5% and 48.6% inhibition at UA concentrations of 60 µg/ml and 30 µg/ml respectively, they also showed that the isolates tested to be resistant through antibiotic sensitivity test to commonly used antibiotics were become sensitive to all the tested antibiotics after treatment with UA at both the tested concentrations. The *S. uberis* isolates treated with UA and resveratrol were more sensitive to antibiotics than untreated. Interestingly, for all the antibiotics used the zone of inhibition or no growth was increased after treating with the antibiofilm agents and the increase in sensitivity of the isolates was directly proportional to the concentration of the antibiofilm agent used. Among the studied antibiofilm agents UA at 100 µg/ml was more effective along with antibiotics than resveratrol. Biofilm inhibiting agents along with all antibiotics had reduced antimicrobial resistance by 1.5-2 times on *in vitro* antibiotic testing by disc diffusion method at 100 µg/ml concentration.

CONCLUSION

The results from this study gives a ray of hope in the treatment of recurrent clinical mastitis by identifying the biofilm forming pathogens. The use of these antibiofilm agents as intramammary infusions has to be studied *in vivo*, since the *in vitro* studies were found to be successful. Anyway, these antibiofilm agents are a hopeful way to tackle the biofilm forming pathogens with existing antimicrobials without any doubt. The extent of increase in sensitivity varies with the combination of antibiotic used and biofilm inhibiting agent selected. UA and resveratrol as an antibiofilm agent, is a promising approach to treat mastitis with the available antibiotics. The studies we conducted are *in vitro* and which has to be done *in vivo* also, that will give more clear data for selection of antibiofilm agents, antibiotics and correlation with the cellular factors.

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