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luxS Gene and Biofilm Formation in *Streptococcus uberis* Isolated from Bovine Mastitis Cases

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ABSTRACT

Background: *Streptococcus uberis* (*S. uberis*) is an environmental pathogen causing mastitis in dairy cattle. It causes recurrent mastitis and reduction in milk production in livestock causing economic loss. The prevalence of *S. uberis* intramammary infections is due to ability of the organism to form biofilm in udder tissue. The present study is on *in-vitro* biofilm production, the correlation of *luxS* gene and the biofilm formation in *S. uberis*.

Methods: A total of 91 mastitic milk samples were collected from cattle and buffaloes brought at Veterinary Hospitals and Farms in Krishna, Guntur and West Godavari districts, Andhra Pradesh. The identification of the culture isolates was based on cultural and biochemical characteristics and confirmed by polymerase chain reaction (PCR). The *Streptococcus* species cultures showing greyish, pinpointed colonies and/or aesculin hydrolysis on Edwards medium 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. Confirmation of the isolates by PCR was followed by detection of biofilm formation using qualitative congo red agar (CRA) method, quantitative microtiter plate (MTP) assay and biofilm gene (*luxS*) was detected using PCR.

Conclusion: From this study it is suggestable that for biofilm study both phenotypic and genotypic methods should be taken together which can be influenced by various other factors also. MTP assay was a good choice for quantitative biofilm determination, which was giving a more accurate and understandable results. The results express that any of the isolates without *luxS* didn't produce a strong biofilm and it is concluded that there may be other genes for regulation of biofilm production and/or *luxS gene* has a regulatory role for one or more genes related to biofilm formation in *S. uberis*.

Key words: Biofilm, Congo red agar, *luxS*, Microtiter plate assay, *Streptococcus uberis*.

INTRODUCTION

S. uberis is a ubiquitous environmental pathogen colonizing and infecting Dairy cattle (De Vos *et al.,* 2009). The dry period is the highly susceptible period to *S. uberis* infection (Marshall *et al.,* 1986). The incidence of new *S. uberis* infections can be reduced by prophylactic antibiotic treatment during dry period (Williamson *et al.,* 1995). Steeneveld *et al.* (2007) reported that *S. uberis*, a primary environmental pathogen was a major cause of mastitis in dairy cattle. Chronic mastitis caused by *S. uberis* is extremely costly and difficult to treat.

Biofilms are formed as a result of the cell multiplication leading to a mature structure consisting of many layers of cells, connected to each by extracellular polysaccharides (Yarwood and Schlievert, 2003). These exopolysaccharides can be enzymes (Mootz *et al.,* 2013; Tielen *et al.,* 2013) or structural proteins (Cucarella *et al.,* 2001) and other polymers (*e.g.*, lipids) (Davey *et al.,* 2003). Although biofilms formed by other *Streptococcus* species like *S. mutans* were well examined and characterised, the ability of *S. uberis* to form biofilms was only recently described (Crowley *et al*., 2011). Merritt *et al.* (2003) identified the *luxS* gene of *S. mutans* which was recognized as the enzyme primarily responsible for the production of autoinducer-2 (AI-2) interspecies quorum signals. The *luxS*-dependent quorum sensing is involved in biofilm formation of *S. mutans*. The detailed microscopic analyses of *luxS* mutant *S. mutant* biofilm indicate a clear difference in mature biofilm structure between the wild type and *luxS* mutants. Yanping, (2017) stated that the virulence of the *luxS* mutant strain of *S. agalactiae* was decreased in the tilapia infection model, exogenous AI-2 molecule and *luxS* gene complementation with plasmid could complement the deficiencies of function in the *luxS* mutant strain.

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MATERIALS AND METHODS

Sample collection

A total of 91 milk samples were collected from mastitis cases of cattle and buffaloes from Veterinary Hospitals and Farms

in Krishna, Guntur and West Godavari districts, Andhra Pradesh. The details of the Veterinary Hospitals and farms are listed in Table 1. The study was carried out during the period of October 2017 to March 2018, in the Department of Veterinary Microbiology, NTR College of Veterinary Sciences, Gannavaram, Andhra Pradesh.

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.

Phenotypic characterization

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.

Table 1: Details of mastitic milk samples collected.

Source of the sample	No. of samples examined
TVCC, Gannavaram	33
VPC, Tanuku	21
VD, Gudavalli	11
VD, Agiripalli	4
Ramavarapadu	13
VD, Sattenapalli	7
Buffalo Research Station, Venkata Ramanna Gudem	2
Total	91

Biofilm detection

The detection of slime production in *Streptococcus* species isolates was done according to Freeman *et al.* (1989). CRA was prepared using BHI agar supplemented with 5% sucrose and 0.08% Congo red dye. The dye was prepared as a concentrated aqueous solution and it was autoclaved at 121°C for 15 minutes separately from the other medium constituents. Congo red dye was added when the agar had cooled to 55°C. The plates were inoculated and incubated at 37°C for 24- 48 hr.

The ability of *S. uberis* strains to form biofilms *in vitro* on an abiotic surface was determined with a method previously described by others (Christensen *et al.,* 1985; Merrit *et al.*, 2005) with minor modifications by Moore, (2009).

Quantification of biofilm producing colonies was done according to Milanov *et al.* (2015). Cut-off OD (ODc) is defined as three standard deviations above the mean OD of the negative control. Strains were classified as follows: Non-biofilm producers (OD ≤ ODc); Weak biofilm producers (ODc<OD≤2×ODc); Moderate biofilm producers (2×ODc <OD≤4×ODc); Strong biofilm producers (OD>4×ODc).

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.

PCR identification and detection of *luxS* gene

The *S. uberis* was confirmed by using species specific primers Sub 302/Sub 396 coding for 23S rRNA and *luxS* gene was detected by specific PCR primer. The primers used in this study and other details are mentioned in (Table 2). The PCR tests were carried out in Proflex PCR system, Applied Biosystems. All the reactions were carried out in a volume of 25 µl in 0.2 ml PCR tubes. The PCR amplicons were analyzed by electrophoresis on 1.7% agarose gel stained with 0.5 µg of ethidium bromide/1 ml in Tris-Borate EDTA (TBE) buffer. The conditions and components of PCR are given in (Table 3 and 4) respectively.

RESULTS AND DISCUSSION

Majority of the *S. uberis* isolates were having good growth in anaerobic environment but few were not, those isolates had a good growth in aerobic environment. So, for those

Table 2: Sequence of primers used for detection of *S. uberis* and *luxS* gene.

Name of primers	Sequence (5′-3′)	Size of the amplified product	Reference
Sub 302 (F)	CGA AGT GGG ACA TAA AGT TA	94 bp	Riffon *et al.,* (2001)
Sub 396 (R)	CTG CTA GGG CTA AAG TCA AT		
luxS (F)	TTT GAT GTT CGC TTG GTT CA	317 bp	Moore 2009
luxS (R)	AGT TTT GCC CAT TCT TTT GC		

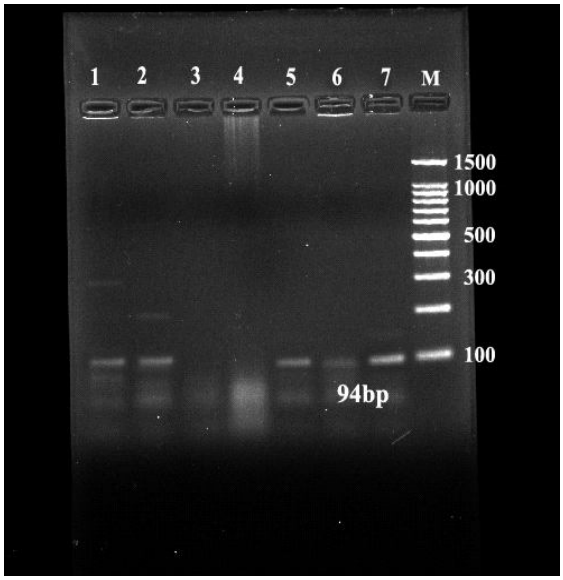
isolates aerobic environment were provided for the further tests. The suspected isolates of *Streptococcus* species were further identified by various biochemical tests *viz.,* negative catalase test, positive aesculin production test, positive sodium hippurate hydrolysis test. The haemolysis pattern observed on 7% sheep blood agar was 86.36% isolates showed α- haemolysis, 2.27% isolates were β- haemolytic and 11.36% isolates were non-haemolytic. Fourty four isolates were found to be *S. uberis* with product size of 94 bp (Fig 1) by PCR.

By using CRA method, 79.54% of the isolates were positive for biofilm production, with black or black colonies with dry consistency (Fig 2) and 20.45% isolates were non biofilm producers (Fig 3). On MTP assay of the isolates 9.09% were strong biofilm producers, 6.8% were moderate biofilm producers, 68.18% were weak biofilm producers and 15.9% were non biofilm formers (Fig 4). In accordance with these findings, Crowley *et al.* (2011) reported 83.33% *S. uberis* strains produced biofilm. The *S. uberis* positive cultures were tested for *luxS* gene by PCR. Fifteen (34.09%) isolates reacted to *luxS* primers with product size of 317 bp (Fig 5). The details of phenotypic ability to form biofilm and the presence of *luxS gene* in each isolate is given in Table 5.

MTP assay was giving consistent results for detecting and quantifying biofilm whereas CRA inconsistent and duplication, required huge number of materials and hard work. In this study *luxS* gene responsible for biofilm production was detected in 34% of isolates by PCR. These findings are consistent with Satish Kumar, (2016) who reported the presence of *luxS* gene in 41.6% of *S. uberis* isolates. In contrary Moore, (2009) reported the presence of *luxS* gene in 96% of isolates. On evaluation of the data obtained, also observed that 24 isolates without *luxS* genes produced very weak or moderate biofilm which may be due to different methods of biofilm growth. This was in association with Wen *et al.* (2002) and Merritt *et al.* (2003) who reported *luxS* mutant of *S. mutans* is still able to form biofilms on solid surfaces. Merritt *et al.* (2003) reported several altered biofilm phenotypes: Increased size of cell aggregates, altered biofilm structure and an increased biofilm resistance to detergents and antibiotics by the *luxS* mutant strains of *S. mutans*. Yadav *et al*. (2018) reported that the expression of the genes involved in virulence and bacterial fitness of *Streptococcus pneumoniae* is regulated by LuxS/AI-2. The SEM revealed thin and scattered biofilms formed by the *luxS* Mutant *S. pnuemoniae* strain in rat model.

Table 3: PCR program. PCR was run for 35 cycles and final extension step was maintained at 72°C for 10 min for all the oligonucleotide primer sets.

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



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 1: PCR amplification product of Sub 302 and Sub 396 oligonucleotide primers for *S. uberis.*

Fig 2: Strong biofilm forming black colonies of *S. uberis.*

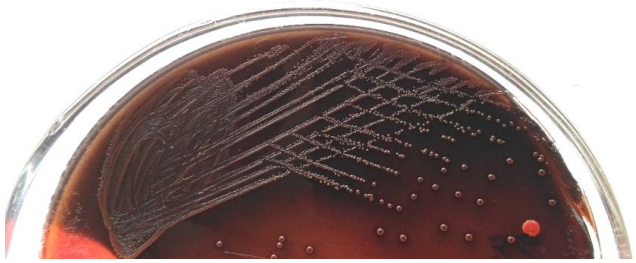


Fig 3: Non biofilm forming colonies of *S. uberis* with blackening at the centre.

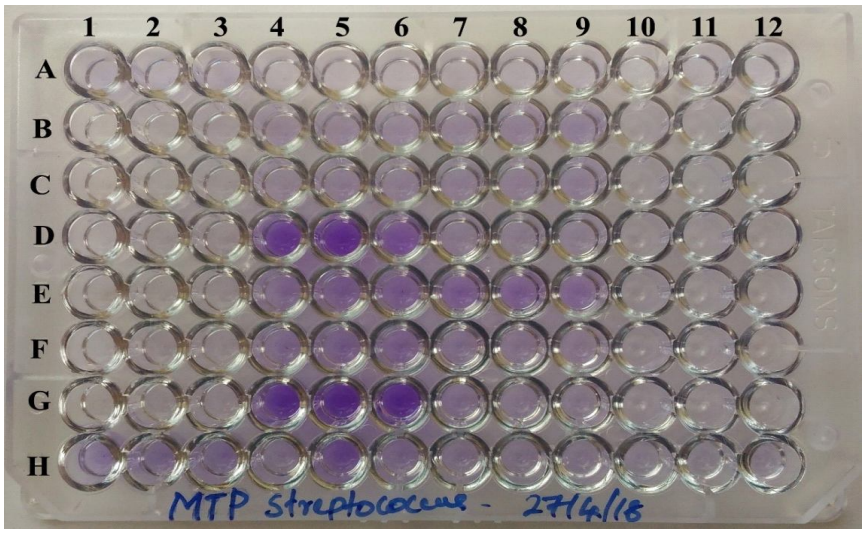
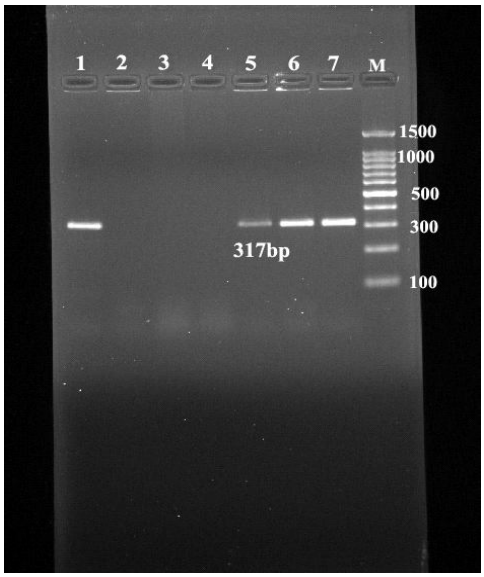


Fig 4: MTP of *S. uberis* isolates, G4 to G6- strong; E4 to E6- moderate; C4 to C6- weak; A1 to A3-non biofilm former and A10 to A12-negative control.



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

Fig 5: PCR amplification product of *luxS* gene of *S. uberis*.

Table 4: Composition of master mix.

Reagents	Quantity (µl)
2X master mix (Promega)	12.5
Forward primer	1.25
Reverse primer	1.25
DNA template	2.5
Distilled water	7.5
Total	25

Table 5: Results of biofilm detection in *S. uberis:* n=44.

Culture no.	CRA	MTP assay	*luxS* gene
2	+	W	_
5	+	W	+
6	+	W	_
26	+	W	+
29	+	W	_
31	+	W	_
32	+	S	_
33	+	W	_
35	_	W	_
36	_	W	_
39	_	W	_
40	_	W	_
41	+	M	+
43	_	W	_
45	+	W	+
46	+	W	_
50	+	S	+
51	+	W	_
53	_	W	_
54	+	W	_
55	+	W	_
56	+	W	_
57	+	W	+
58	+	S	_
59	_	NB	+
60	+	W	+
62	+	W	_
63	+	M	+
64	+	NB	_
65	+	W	+
67	_	W	_
69	+	NB	_
70	_	S	_
72	+	NB	_
76	+	NB	+
77	+	W	_
79	+	NB	_
80	+	M	_
82	+	W	+
83	+	W	_
84	+	NB	_
85	+	W	+
87	+	S	+
91	+	S	+

S- strong biofilm; M- moderate biofilm; W- weak biofilm; NB- non biofilm former; + -detected; _- not detected.

CONCLUSION

From this study it is suggestable that both phenotypic and genotypic methods should be taken together for determination of biofilm production, which can be influenced by various other factors. Also, the results express that any of the isolates without *luxS* didn't produce a strong biofilm and it is concluded that there may be other genes for regulation of biofilm production and/or *luxS gene* has a regulatory role for one or more genes related to biofilm formation in *S. uberis*.

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