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Detection of biofilm formation ability of *Streptococcus agalactiae* isolated from bovine mastitis cases

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ABSTRACT

Bacterial biofilms are thought to be the predominant growth mode in their natural environments and increasing evidence implicates biofilm as the cause of various animal infections. In this study biofilm forming ability was demonstrated in *Streptococcus agalactiae* isolated from bovine mastitis using 96-well microtiter plates. Twelve isolates were screened for biofilm formation using Luria-Bertani (LB) medium with one per cent glucose. Twenty five per cent of the isolates were strong biofilm producers with OD values of $\geq$1.0, 16.67 per cent were moderate biofilm producers with OD values between 0.5 and 1 and 58.33 per cent were considered as non-biofilm producers with OD value of <0.5. The influence of addition of glucose on *S agalactiae* biofilm formation was studied by subjecting all the isolates for biofilm formation using LB medium with and without glucose. In LB glucose medium the similar results were obtained as above but in LB without glucose medium only 16.67 per cent isolates were moderate biofilm producers and 85.33 per cent did not produce biofilms. Biofilm forming ability of *S agalactiae* was also studied in Todd-Hewitt broth (THB) and tryptone soya broth (TSB) media supplemented with 1 per cent glucose. In case of THB glucose only 8 and 17 per cent of the isolates produced strong and moderate biofilms respectively whereas 75 per cent of the isolates were non-biofilm producers. In TSB glucose only 8 per cent of the isolates produced strong biofilms and 92 per cent were non-biofilm producers. This study suggests that *S agalactiae* of bovine origin is a biofilm producer and the type of medium and glucose concentration influence its biofilm production.

Keywords: Mastitis; bovine; *Streptococcus agalactiae*; biofilm; kinetics

INTRODUCTION

Streptococcus species are one of the most important groups of causatives of mastitis in bovines. *Streptococcus agalactiae* is an obligate parasite of the bovine mammary gland with herd prevalence rates ranging from 11 (16) to 47 per cent (Goldberg et al 1991). In vitro studies have shown that *S agalactiae* isolated from both animal and humans are potential biofilm producers (Rinaudo et al 2010, Konto-Ghiorghi et al 2009, Olson et al 2002). Earlier these microorganisms were studied by culturing them in highly enriched liquid or solid media. However bacteria existing within natural systems are entirely different from artificially grown laboratory strains. Sessile bacteria growing on surfaces have nutrient limitations and so growing more slowly whereas planktonic bacteria in culture media have unnatural access to nutrients, multiply rapidly and often are highly motile. Hence planktonic bacteria are more susceptible to the effects of antibiotics and environmental and host factors. Conversely sessile bacteria are able to resist or evade such destructive factors by forming aggregates, altering their physiology and taking advantage of deficiencies in the host clearance mechanisms (Costerton et al 1995, Mah and O'Toole 2001).

Many persistent and recurrent infections have been attributed to the formation of biofilm or polymeric

matrices produced by bacterial colonies adhering to a biologic or abiotic surface. A biofilm matrix is composed of microbial cells, polysaccharides, water and other extra-cellular products all of which allow the biofilm matrix to be hostile to numerous micro-environments (Costerton et al 1999, Mah and O'Toole 2001, Sutherland 2001).

Biofilms are a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface. Nearly 99 per cent of microorganisms found on the earth live in microbial communities known as biofilms (Costerton et al 1999). As per the available literature on screening of biofilm forming ability of bacteria, polystyrene microtiter plate serves as a good inert surface for attachments, colonization and biofilm formation. The microtiter plate assay is an important tool to study early stages of biofilm formation and has been applied primarily for the study of bacterial biofilms (Konto-Ghiorghi et al 2009, O'toole 2011). Hence the biofilm forming ability of *S agalactiae* was studied using 96-well microtiter plates. At the same time the influence of glucose and different media was studied on biofilm forming ability of *S agalactiae.*

MATERIAL and METHODS

***S agalactiae* isolates:** Twelve *S agalactiae* isolates isolated from bovine mastitis cases and maintained at Department of Veterinary Microbiology, Veterinary College, Bangalore, Karnataka were used.

Screening of *S agalactiae* isolates for biofilm forming ability: Initial inoculum of *S agalactiae* was prepared as per the method described by Konto-Ghiorghi et al (2009). *S agalactiae* was grown in Todd-Hewitt broth (THB) for 18 hour; then two-fold serial dilution was made in fresh Luria-Bertani (LB) broth with one per cent glucose (LB glucose) using 96-well microtiter plates. The absorbance was recorded at 620 nm using ELISA plate reader and optical density (OD) value of dilution showing 0.1 absorbance was selected for each isolate for further processing.

Biofilm forming assay: *S agalactiae* isolates were screened for biofilm forming ability in 96-well microtiter plates following the method described by Konto-Ghiorghi et al (2009).

Growing of *S agalactiae* biofilm: *S agalactiae* grown in THB for 18 hour at 37°C under 5 per cent CO_2 tension was diluted in LB glucose medium to obtain the OD value of 0.1 at 620 nm. The diluted culture was vortexed briefly and 180 µl was dispensed into 96-well microtiter plate (Nest tissue culture plates, sterile 96-well from M/s Tarsons Products Pvt Ltd, Kolkata, West Bengal, India) in triplicates with maintaining the fourth well as negative control with medium only. The lid was kept on the plate and sealed with parafilm and the plate was incubated in static condition at 37°C for 24 hour under 5 per cent CO_2.

Staining of biofilm: After 24 hour planktonic cells along with medium were dumped out by turning the plates upside down; the plates were washed twice in PBS and air-dried for 15 min. Hundred µl of 0.1 per cent (w/v) crystal violet (CV) stain was added to each well and the plate was incubated for 30 minute at room temperature. After staining the plate was washed twice with PBS and air-dried for few hours or overnight at room temperature by keeping the plate upside down. After drying the bacterial biofilms in the bottom and walls of the plate was recorded by digital camera and inverted microscope.

Quantification of biofilm: One hundred and twenty five µl of ethanol/acetone (80:20) was added to each well to solubilize the CV and the plate was incubated at room temperature for 10-15 minute and absorbance was measured at 595 nm using ELISA plate reader (M/s BIORAD-680, USA).

Effect of glucose on *S agalactiae* biofilm formation: All the 12 *S agalactiae* isolates were screened for biofilm forming ability in 96-well microtiter plates in LB with 1 per cent glucose and LB without glucose following the above mentioned method.

Effect of different media on *S agalactiae* biofilm formation: All the 12 *S agalactiae* isolates were screened for biofilm forming ability in 96-well microtiter plates in three different media such as LB, THB and tryptone soya broth (TSB) supplemented with 1 per cent glucose following the above mentioned method.

Statistical analysis: Statistical analysis was carried out using the statistical software GraphPad Prism version 5 for windows. The data were analysed by two-way ANOVA.

RESULTS

***S agalactiae* biofilm forming ability:** To assess the capacity of *S agalactiae* to form biofilm twelve isolates were subjected for biofilm formation. Among twelve isolates screened for biofilm formation, three (SA3, SA5 andSA6) were considered as strong biofilm producers with OD value of $\geq$1.0, two (SA1 and AD1) were considered as moderate biofilm producers with OD values between 0.5 and 1 and the remaining seven (SA2, SA4, SA7 through SA11) isolates were considered as non-biofilm producers with OD value of <0.5 (Table 1, Fig 1, Plates 1, 2, 3, 4, 5).

Influence of glucose on *S agalactiae* biofilm formation: An attempt was made to study the influence of addition of glucose on the ability of *S agalactiae* to produce biofilms. For this purpose LB medium with and without one per cent glucose was used. In LB glucose medium three (25%) isolates were strong, two isolates (16.67%) were moderate and the remaining (58.33%) isolates were non-biofilm producers. Whereas in LB without glucose medium only two isolates (16.67%) were moderate biofilm producers and the remaining (84.33%) isolates did not produce biofilms (Figs 2, 3).

Influence of different media on *S agalactiae* biofilm formation: While studying the influence of different growth media on biofilm forming ability of *S agalactiae* it was found that in THB glucose medium only one produced strong biofilms, two produced moderate biofilms and remaining isolates were non-biofilm producers. In TSB glucose medium out of twelve isolates only one produced strong biofilms and remaining isolates did not produce biofilms (Table 2, Fig 3).

It was found that the biofilm production was significantly ($P < 0.001$) high in LB compared to THB and TSB glucose medium.

DISCUSSION

Mastitis is a complex disease having different etiology and degrees of intensity and variations in duration and residual effects. Among the mastitis causing bacterial agents *S agalactiae* is one of the most predominant pathogens causing clinical mastitis. In recent days the number of mastitis cases not responding to generally used antibiotics therapy are increasing.This could be attributed to frequent and indiscriminate use of antibiotics, emergence of drug resistant *S agalactiae* strains besides the potential biofilm forming ability of this organism. The contribution of biofilm forming ability to complexity of such bacterial infection has been extensively studied (Costerton et al 1995, Mah and O'Toole 2001) and one of the most convincing hypotheses to explain therapeutic resistance is the ability of many bacterial infections to grow as biofilm in infected tissues thus developing an innate resistance to almost all therapeutic agents.

Screening of *S agalactiae* isolates for biofilm formation: Surface attachment and biofilm forming ability in 96-well polystyrene microtiter plates were studied in *Pseudomonas fluorescens* (O'Toole and Kolter 1998), *S gordonii* and 15 other oral streptococci (Loo et al 2000), *S mutans* (Zezhang and Robert 2002), *Enterococcus faecalis* (Fabretti et al 2006) and *S agalactiae* (Konto-Ghiorghi et al 2009, Kaur et al 2009, Rinaudo et al 2010).

The biofilm formation in *S agalactiae* was analyzed based on the absorbance of the crystal violet stained biofilm at A_{550} and the OD of 0.5 was taken as the cut-off point to know the biofilm forming ability of *S agalactiae* and to differentiate between biofilm forming and non-forming isolates. These findings are in conformity with the findings of Kaur et al (2009) who also reported that *S agalactiae* isolates were considered as good ($A_{550} \geq 1.0$), moderate ($A_{550} \geq 0.5$-1.0) and poor ($A_{550} < 0.5$) biofilm formers on the basis of their absorbance property. Whereas Loo et al (2000) categorized the *S gordonii* strains as good biofilm formers when the absorbance at 575 nm of the CV stained biofilms was greater than 2.0. On the other hand Mathur et al (2006) reported OD values greater than 0.24 as indicator of strong biofilm production in *Staphylococcus* spp in CV stained and dried biofilms without adding the diluents at A_{570}.

Influence of glucose on *S agalactiae* biofilm formation: Recent studies have shown that the addition of glucose is necessary for growing bacteria in biofilm mode. Mathur et al (2006) investigated the biofilm formation by clinical isolates of *Staphylococcus* spp and reported that in TSB medium only 4.6 tested *Staphylococcus* isolates displayed a biofilm positive phenotype while in the presence of glucose biofilm production increased to 52.6 per cent. Similarly other investigators also studied the biofilm formation in various bacterial species such as *Pseudomonas aeruginosa*, *P fluorescens, Klebsiella pneumonia* and

Table 1. Screening of *S agalactiae* isolates for biofilm forming ability

Experiment	Average OD values of *S agalactiae* biofilms in microtiter plates											
	SA1	SA2	SA3	SA4	SA5	SA6	SA7	SA8	SA9	SA10	SA11	AD1
First	1.07	0.42	2.20	0.45	1.88	1.74	0.44	0.52	0.45	0.47	0.44	0.70
Second	0.87	0.40	1.79	0.46	1.44	1.46	0.45	0.46	0.44	0.48	0.44	0.99
Third	0.95	0.42	2.22	0.47	1.90	1.75	0.51	0.48	0.47	0.46	0.45	0.70
Mean	0.96	0.41	2.07	0.46	1.74	1.65	0.47	0.49	0.46	0.47	0.45	0.80

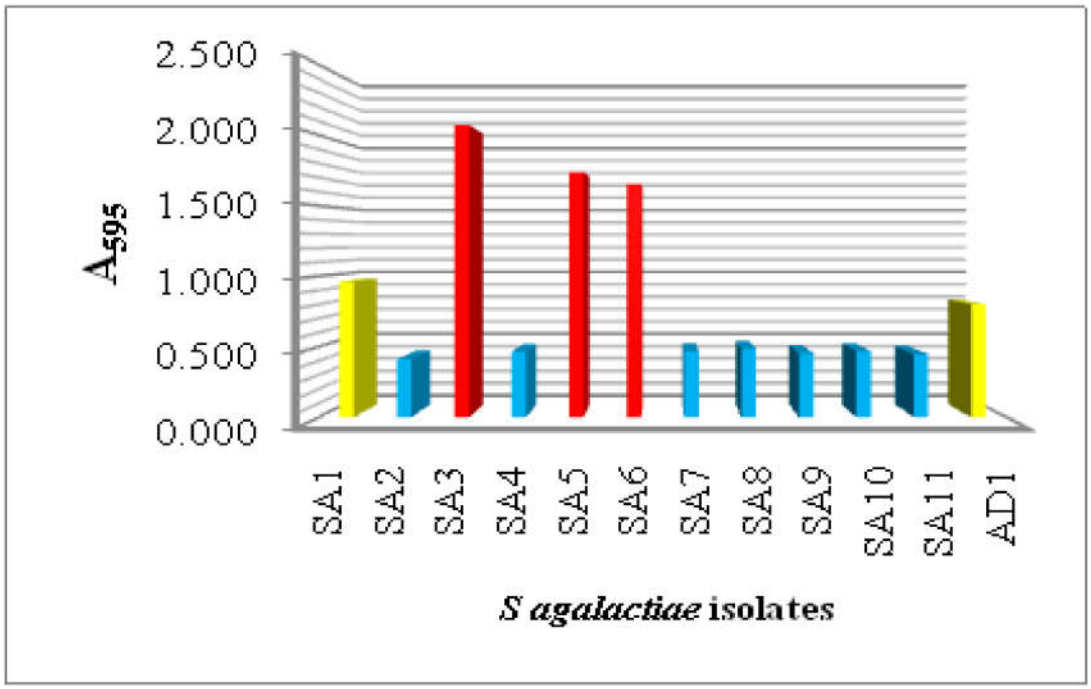

Fig 1. Biofilm formation by different isolates of *S agalactiae*

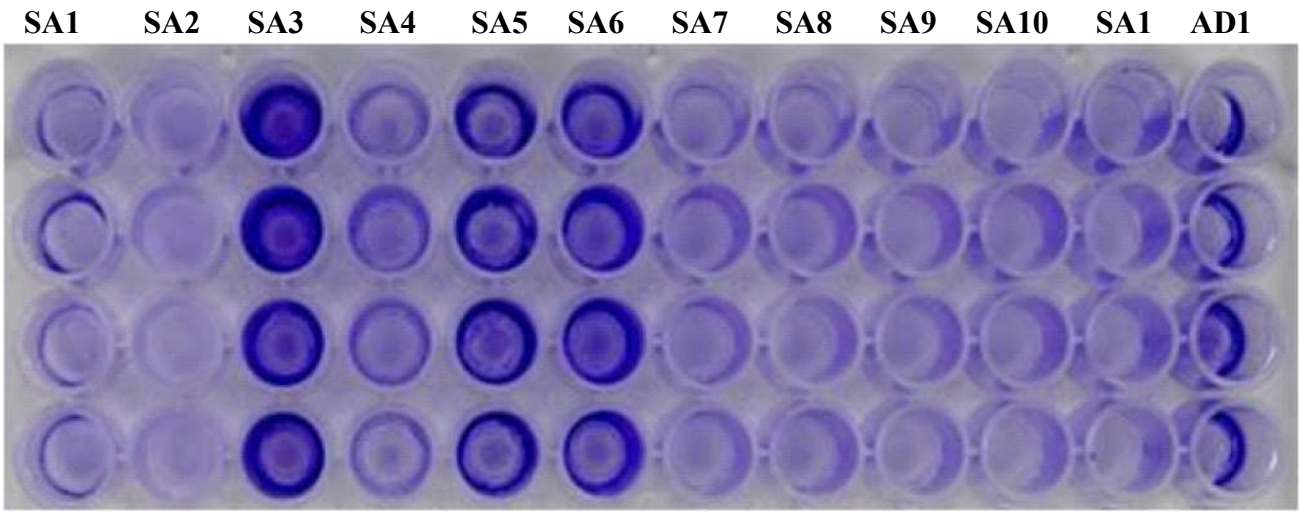

Plate 1. Microtiter plate showing biofilm formation

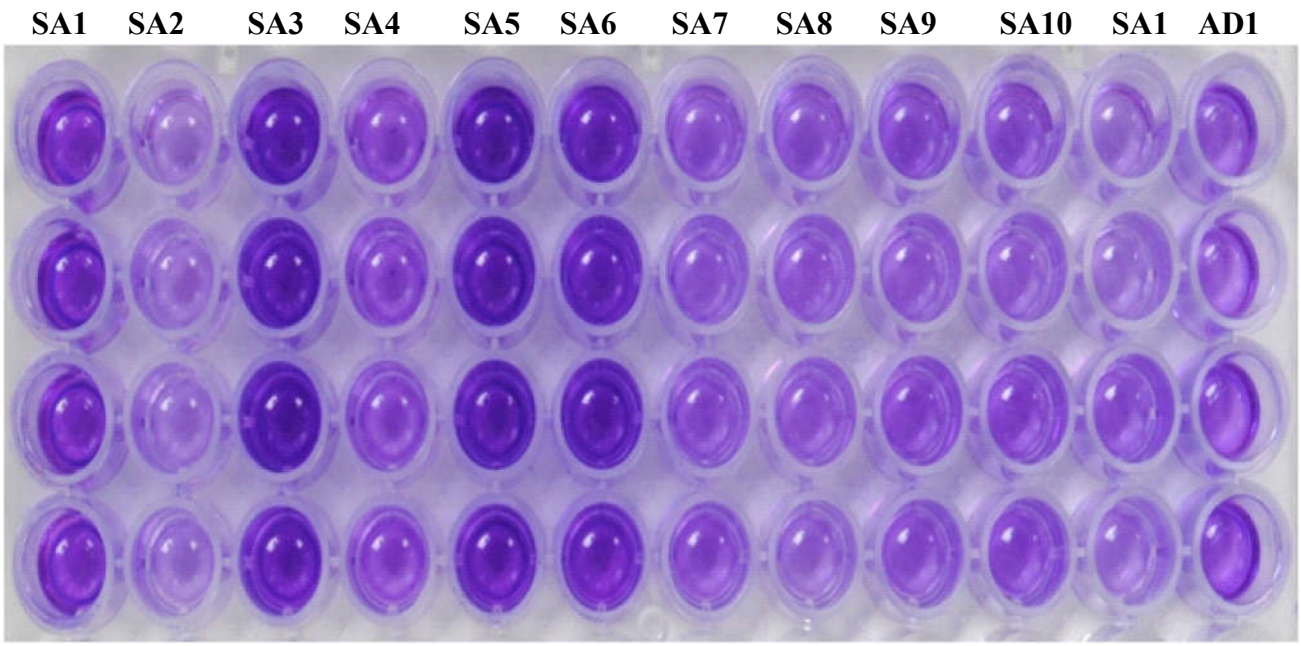

Plate 2. Microtiter plate showing biofilm formation after adding the diluents

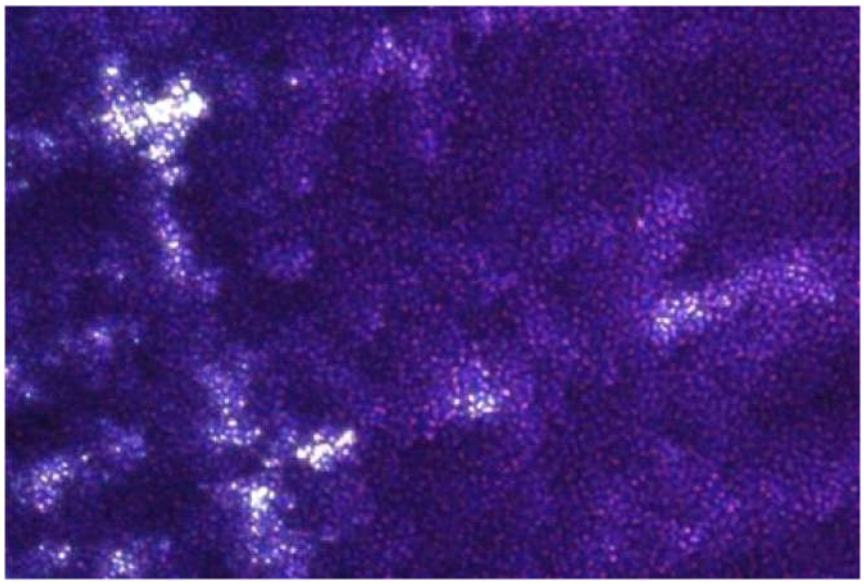

Plate 3. Micrograph of *S agalactiae* SA3, strong biofilm producer (400X)

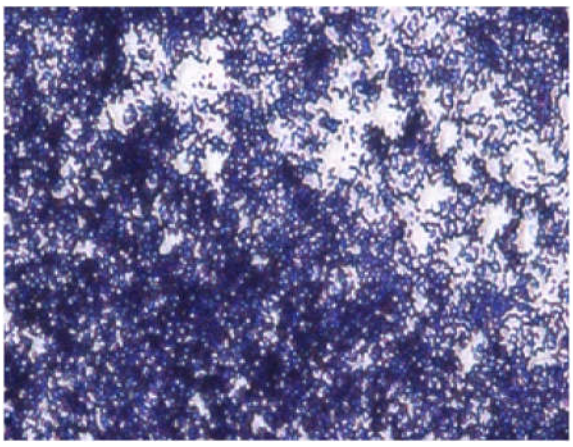

Plate 4. Micrograph of *S agalactiae* SA1, moderate biofilm producer (400X)

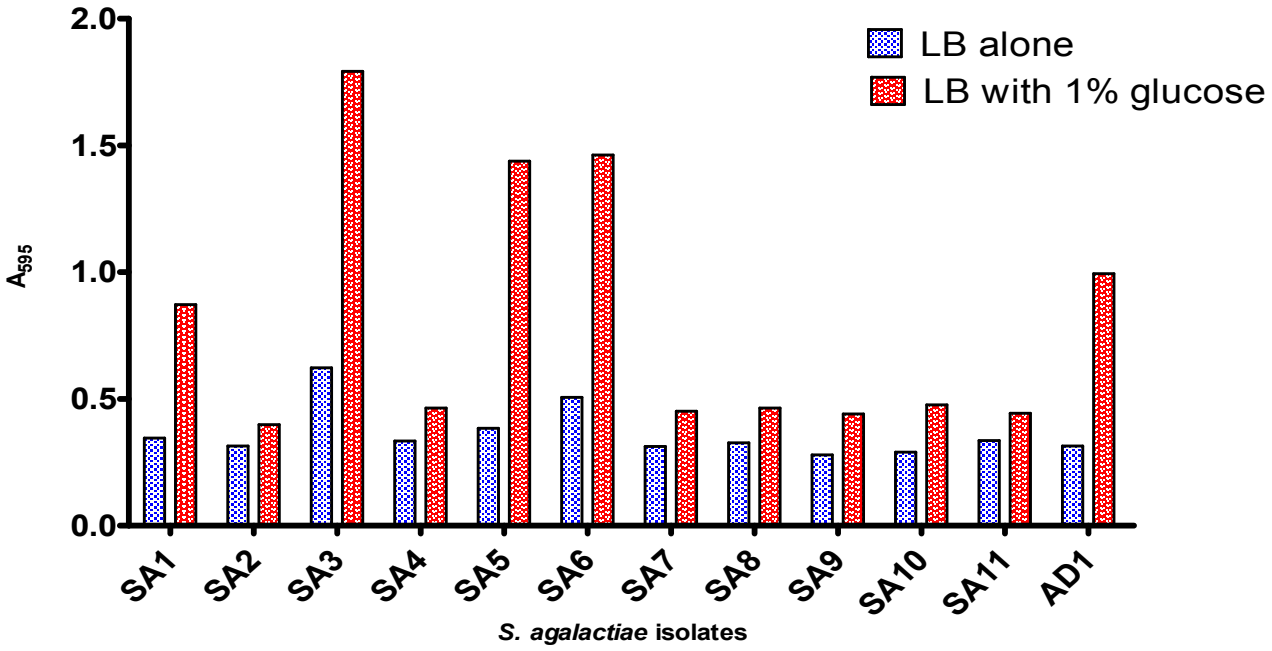

Fig 2. Influence of glucose on *S agalactiae* biofilm

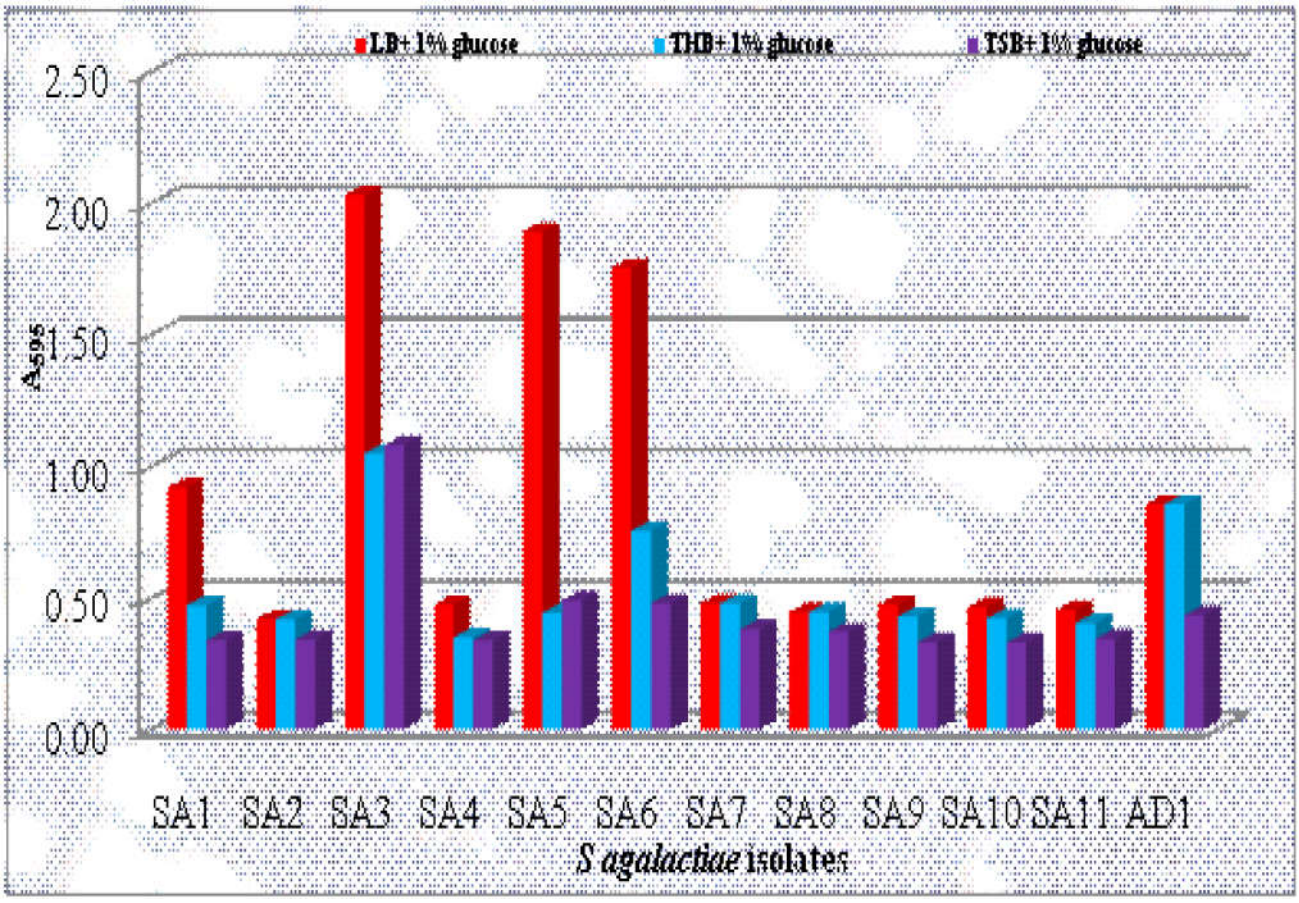

Fig 3. Influence of different growth media on *S agalactiae* biofilm formation

Table 2. Influence of different growth media on *S agalactiae* biofilm formation

Medium	Average OD values of *S agalactiae* biofilms in microtiter plate											
	SA1	SA2	SA3	SA4	SA5	SA6	SA7	SA8	SA9	SA10	SA11	AD1
LB glucose	0.92	0.42	2.03	0.47	1.89	1.75	0.48	0.45	0.47	0.46	0.45	0.85
THB glucose	0.47	0.42	1.05	0.35	0.44	0.75	0.48	0.45	0.43	0.42	0.40	0.85
TSB glucose	0.34	0.34	1.08	0.34	0.49	0.48	0.38	0.37	0.33	0.33	0.34	0.43

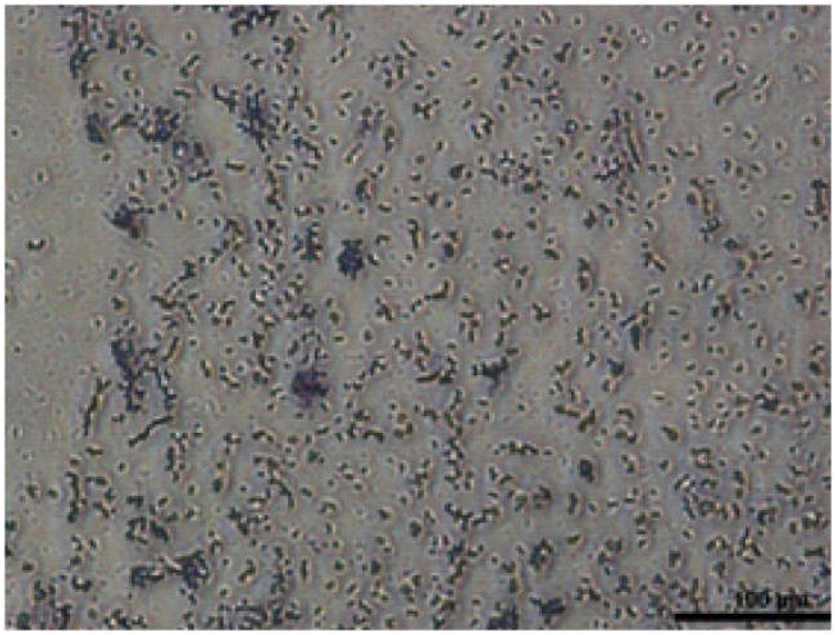

Plate 5. Micrograph of *S agalactiae* SA7, non-biofilm producer (400X)

Stenotrophomonas multophilia isolated from environmental and industrial biofilms (Stoodley 1999) and different isolates of *S agalactiae* (Stoodley et al 2002, Kaur et al 2009, Rinaudo et al 2010) in various media supplemented with glucose.

The study showed that *S agalactiae* isolated from the cases of bovine mastitis were poor biofilm producer in the absence of additional one per cent glucose whereas in the presence of one per cent additional glucose the biofilm formation ability was significantly high.

Influence of different growth media on *S agalactiae* biofilm formation: The composition of growth medium greatly influences biofilm formation in bacteria. Under the present investigations *S agalactiae* biofilm forming ability was studied in three different growth media viz LB, THB and TSB media supplemented with one per cent glucose using microtiter plate method.

It appears that nutritionally rich environment does not favour *S agalactiae* biofilm formation on polystyrene plates but nutritionally limited environment increases the growth of sessile bacteria. The comparison between the growth conditions revealed that the restriction of nutrients was must for *S agalactiae* to form biofilms. As fas as the chemical composition of the three different media is concerned LB medium is the least enriched medium compared to THB and TSB indicating that the ideal medium for *S agalactiae* biofilm formation is LB supplemented with one per cent glucose. These findings are in conformity with those of Konto-Ghiorghi et al (2009) who reported that LB medium supplemented with one per cent glucose produced uniform biofilms on the polystyrene microtiter plates.

During biofilm mode of growth the initial attachment of bacteria to a surface is a necessary step in biofilm formation. The complex pathway leading to biofilm development in different species of microorganisms involves the contribution of growth conditions, surface materials and genetic factors. Numerous genes or factors have been identified as being essential or required for biofilm formation in various bacteria (Stoodley et al 2002). Such genes include those that regulate surface-exposed proteins, appendages such as pili or fimbriae and extracellular polymeric substance (EPS) matrix materials. Pili seem to play a key role in adhesion and attachment to host cells both in Gram-negative and Gram-positive pathogens. Their involvement in the transition from planktonic growth to a surface-attached multicellular community has also been demonstrated in many studies (Branda et al 2005).

Recently the involvement of pili in adherence and biofilm formation of *S agalactiae* strains has been well studied and increasing evidences indicate that in pathogenic streptococci, biofilm formation is mediated by pili (Konto-Ghiorghi et al 2009, Rinaudo et al 2010). The distribution and conservation of pili have been characterized in 289 *S agalactiae* clinical isolates by Rinaudo et al (2010). The results showed that *S agalactiae* has three types of pili viz type 1, 2a and 2b encoded by three corresponding pilus islands and that each strain carries one or two islands. They also investigated the capacity of these strains to form biofilms and found that most of the biofilm-formers carried pilus 2a using insertion and deletion mutants further confirming that pilus type 2a but not pilus types 1 and 2b conferred biofilm-forming phenotype. In the current study strong biofilm formation only by *S agalactiae* SA3 in LB, THB and TSB with one per cent glucose might be due to the presence of pili 2a, a surface appendage of *S agalactiae* but that needs to be further elucidated.

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